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Bruno regulates mRNA translation by binding to multiple sequence motifs

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Bruno regulates mRNA translation by binding to multiple sequence motifs

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Dedication

For my parents

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Bruno regulates mRNA translation by binding to multiple sequence motifs

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Oskar (Osk) is a posterior body patterning determinant in *Drosophila melanogaster* oocytes. *oskar* (*osk*) mRNA is translationally repressed until it reaches the posterior of the oocyte where Osk protein accumulates. Translational repression of *osk* prior to posterior localization is mediated by the RNA binding protein, Bruno (Bru). To better define Bru binding sites, I performed in vitro selections using full length Bru and the fragments containing either the first two RRMs (RRM1+2) or the third RRM (RRM3+). The aptamers from the final round from each of the selections produced a multitude of overrepresented primary sequence motifs. Examples of each of these motifs were found in the 3'UTRs of the mRNAs that Bru is known to regulate during oogenesis. GFP reporter transgenes under the control of the UAS-Gal4 expression system were constructed with each class of the binding sites within the reporter transgenes' 3'UTRs to test the motifs' ability to repress the reporters in vivo. In a wildtype background, the GFP reporters containing the binding sites were translationally repressed. In the *aret*

mutant background, the GFP levels of the repressed GFP reporters increased with reduced Bru activity, suggesting the transgenes' repression is mediated by Bru. Three of the motifs isolated in the in vitro selections reside in the AB and C regions of the *osk* 3'UTR, and the three classes of sites were mutated in the AB and C regions. The mutated AB and C regions were used to assay for a reduction of Bru binding affinity for the mutant RNAs. Additionally, the mutations were incorporated into an *osk* genomic transgene that was introduced into an *osk* RNA null as well as an Osk protein null background. The mutations reduced Bru binding to the AB and C regions. The transgenes containing the mutated Bru binding sites could not fully rescue the *osk* RNA null phenotype but can fully rescue the Osk protein null phenotype, suggesting an *osk* transcript can regulate other *osk* mRNAs in trans.

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Chapter 1: General Introduction

LOCALIZED TRANSLATION IN EUKARYOTES

Localization of mRNAs and localized translation are commonly used in eukaryotic species to achieve localized deployment of proteins. In yeast, ASH1 is a transcriptional repressor that inhibits mating type switching. ASH1 mRNA is localized to the bud tip of a dividing yeast cell to ensure ASH1 is translated only in the daughter cell (Paquin and Chartrand, 2008). In fibroblasts, β-actin mRNA is localized to the lamellipodia, where localized translation of β-actin is required for cytoskeletal-mediated motility (Condeelis and Singer, 2005). In the developing and mature mammalian nervous system, localized translation of transcripts aids in the growth and plasticity of neuronal cells (Smith, 2004; Lin and Holt, 2007; Martin and Zukin, 2006). During *Xenopus* oogenesis, the T-box transcription factor, VegT, localizes to the vegetal pole of the oocyte and induces endodermal and mesodermal cell fates in the developing embryo (King et al., 2005). Similarly, during *Drosophila* oogenesis, the basic body plan is determined via localized transcripts, which are locally translated (Johnstone and Lasko, 2001).

Locally translated mRNAs are transcribed in the nucleus where they are processed and packaged into ribonucleoprotein (RNP) complexes. RNPs are exported to the cytoplasm and transported to the RNPs' final destinations with the transport being dependent on filamentous actin or mictrotubules. Once the RNP is localized, the RNA is released from repression and translated into its encoded protein. The only known examples of release from repression involve a localized kinase phosphorylating the translational repressor bound to the locally translated mRNA. Once phosphorylated, the

repessor dissociates from the regulated mRNA, which allows the mRNA to be translated. In yeast and in filapodia, the kinase is an inter-membrane protein localized to the cortex. In dendrites, a signal outside of the cell induces the activation of cytosolic kinases within the region of signaling (Besse and Ephrussi, 2008). It is as yet unknown how translation is activated in the *Xenopus* and *Drosophila* transcripts studied.

MATERNAL CONTRIBUTIONS TO ZYGOTIC DEVELOPMENT

Early *Drosophila* embryo development is controlled by maternal mRNAs and proteins that are loaded during oogenesis (Davidson, 1986). In contrast, embryonic patterning in the mammalian system is dependent on signaling between different cell types for the formation of the body plan (Gilbert, 2010). Oogenesis is the development of the egg and occurs in the ovaries of the female. An average *Drosophila* ovary holds 16 ovarioles (King, 1970). Each ovariole contains 6 to 7 developing egg chambers. The anterior of the ovariole consists of germ-line and somatic stem cells, which differentiate into egg chambers within a region called the germarium. The egg chambers mature and grow as they move toward the posterior of the ovariole until they are competent for fertilization.

Egg chambers are made up of 16 germ cells surrounded by a layer of somatic follicle cells. Germ-line stem cells asymmetrically divide to form a regenerating germ cell and a cystoblast. Egg chambers are formed from the cystoblast, which goes through four mitotic divisions with incomplete cytokinesis to create a 16-cell cyst connected by cytoplasmic bridges called ring canals. 15 of the germ cells will become polyploid nurse cells, while the remaining germ cell will be designated the developing oocyte. Nurse cells provide the RNA, protein, membranes, and organelles that the oocyte needs for

proper development. Transport to the oocyte, of the RNAs and proteins, has been demonstrated to be dependent on microtubules (Pokrywka and Stephenson, 1995). During oogenesis, the oocyte goes through two polarization steps mediated by Gurken (Grk) (González-Reyes et al., 1995). The first step designates the anterior-posterior axis, which occurs between stages 5 and 7 of oogenesis. The second step occurs at stage 8 and designates the dorsal-ental axis. Polarization allows for some of the egg contents to be distributed asymmetrically, which in turn, allows for the establishment of the fly's basic body pattern (van Eeden and St Johnston, 1999; Reichmann and Ephrussi, 2001; Roth and Lynch, 2009).

MATERNAL EFFECT GENES ARE REQUIRED FOR PROPER BODY PATTERNING

Many maternal-effect genes, some of which are required for proper body patterning, have been identified genetically (Fröhnhofer and Nüsslein-Volhard, 1986; Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Schüpbach and Wieschaus, 1989). Mothers carrying mutations in some of these genes can produce embryos with an abnormal body pattern (Schüpbach and Wieschaus, 1989). A subset of these maternal patterning genes encode spatial determinants. Spatial determinants are localized to specific regions in the egg or embryo and are required for proper body patterning of the region. Mislocalization of the determinants to other regions will change the patterning of the region (Driever et al., 1990; Ephrussi and Lehmann, 1992; Frohnhöfer et al., 1986; Gavis and Lehmann, 1992; Webster et al., 1994).

An example of one of these determinants is *oskar* (*osk*). *osk* is a posterior body patterning determinant (Lehmann and Nüsslein-Volhard, 1986). Mothers homozygous for a strong *osk* mutant allele produce embryos that are defective in posterior patterning

and have no abdominal segments. When there is too much or ectopically localized Osk protein, the embryo loses anterior structures, and in extreme cases, a mirror image of the posterior forms at the anterior resulting in the bicaudal phenotype (Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992; Smith et al., 1992).

AN OVERVIEW OF *OSK* LOCALIZATION AND TRANSLATION DURING OOGENESIS

osk mRNA is synthesized in the nurse cell nuclei and forms an RNP with proteins that bind the *osk* mRNA cotranscriptionally (Kugler and Lasko, 2009). *osk* is then transported to the nurse cell cytoplasm. From the nurse cell cytoplasm, *osk* mRNA is transported to the oocyte, a process dependent on microtubules (Pokrywka and Stephenson, 1995). From oogenesis stage 1 to stage 8, *osk* is highly concentrated in the oocyte. At stage 8, the oocyte begins to expand, and *osk* is transiently concentrated at the oocyte anterior. By stage 9, the *osk* message is tightly localized to the posterior of the oocyte and is localized to the posterior until early embryogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). Upon localization of the *osk* message to the posterior, Osk protein is translated (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Osk protein has two isoforms that are produced by using in frame, alternative start codons within the coding region of the message (Markussen et al., 1995). The two isoforms have different activities during development. Long Osk is required for anchoring of RNAs and proteins at the oocyte posterior. Short Osk is required for the formation of the germ plasm, which is required for proper posterior patterning and formation of the precursors to the germ cells, the pole cells (Markussen et al., 1995, Vanzo and Ephrussi, 2002).

Proper *osk* localization and translational regulation is dependent on a complex regulatory network, including a number of proteins that interact with the *osk* message as

it is transported through different regions of the egg chamber. The *osk* message is associated with sponge bodies. Sponge bodies are large RNPs containing post-transcriptional regulatory factors (Wilsch-Brauninger et al., 1997). Sponge bodies reorganize when they travel from the nurse cell cytoplasm to the oocyte with the components of the sponge bodies changing upon entering the oocyte (Mische et al, 2007; Snee and Macdonald, 2009). This could be an indication that the *osk* RNP is a dynamic particle that reorganizes in each region of the egg chamber. The proteins that interact with *osk* in the different regions of the egg chamber enable proper localization of the *osk* message to the oocyte posterior, as well as translational repression of the transcript as it is in transit. An additional group of proteins are involved in the translational activation of the *osk* transcript when it reaches to the oocyte posterior.

OSK MRNA TRANSPORT

osk transport to the posterior is dependent on microtubules (Pokrywka and Stephenson, 1995). Prior to stage 7, microtubules in the oocyte are nucleated from a microtubule organizing center (MTOC) at the posterior. After the Grk signal at the oocyte posterior establishes anterior-posterior polarity in the oocyte, the microtubules in the oocyte reorganize. The MTOC is disassembled, and the microtubule minus ends nucleate from the oocyte's anterior and lateral cortex. This creates a gradient where the concentration of microtubules at the anterior is greater than at the posterior. In addition, it is thought the microtubule plus ends are directed toward the oocyte posterior. While the microtubule reorganization is occurring in the oocyte, the polarity of the microtubules in the nurse cell cytoplasm remains same (Steinhauer and Kalderon, 2006).

Motor proteins can attach and move along the microtubules in either a minus end directed or a plus end directed fashion. The minus end directed microtubule motor, Dynein, is required for *osk* transport into the oocyte cytoplasm but not for *osk*'s localization to the posterior pole (Januschke et al., 2002). The plus end directed microtubule motor protein is Kinesin (Kamal and Goldstein, 2002). In a Kinesin heavy chain mutant (khc^{27}), *osk* is properly localized to the oocyte but posterior localization is absent. The khc^{27} mutant does not alter the localization of the anterior body patterning determinant, *bicoid*, or anterior accumulation of *osk* in stage 8 oocytes (Brendza et al., 2000). From the combined data, *osk* seems to switch from Dynein mediated movement in the nurse cells to Kinesin mediated movement in the oocyte.

Additional support for this model comes from live imaging of *osk* mRNA in the ooctye. An *osk* mRNA was constructed containing MS2 binding sites. The *osk*-MS2 RNA was expressed in flies also expressing an MS2-nls-GFP fusion protein to track the *osk*-MS2 RNA's movement in the ooctye. In the oocyte post stage 9, *oskMS2*/MS2-nls-GFP particles show a weak posterior bias, which is due to plus end directed microtubule movement (Zimyanin et al., 2008). Mutants in the plus end directed microtubule motor, Kinesin, reduce the speed of the anterior and posterior *osk* mRNA particle movements in the oocyte, implicating Kinesin as the motor protein responsible for *osk* transport in the oocyte (Zimyanin et al., 2008).

Movement of mRNA in the egg chamber is either through cytoplasmic flows mediated by the microtubule motor proteins or direct interaction with the microtubule motor proteins. Microtubule motor proteins have not been shown to bind RNA. Therefore, transport requiring mRNA interaction with the microtubule motor proteins would require an intermediate to link the microtubule motor proteins with the mRNAs being transported. A candidate protein complex that could link mRNAs to motor proteins

is the Exon Junction Complex (EJC). The EJC is a multi-protein complex that binds mRNA 5' of exon-exon junctions when the RNA is spliced, and the core proteins of the EJC in *Drosophila* are Mago Nashi, Y14/Tsunagi, Barentsz (Btz), and eIF4AIII (Mohr et al., 2001; Palacios et al., 2004). In other systems, the EJC has been linked to splicing, greater translational efficiency, nuclear export, and nonsense-mediated decay (Tange et al., 2004). In *Drosophila*, the EJC is also involved in proper localization of the *osk* transcript. *mago nashi* and *y14/tsunagi* mutants disrupt *osk* localization to the oocyte posterior (Newmark and Boswell, 1994; Mohr et al., 2001; Hachet and Ephrussi, 2001). In addition, an eIF4AIII mutant genetically interacts with *barentz* (*btz*) to abrogate *osk* localization to the posterior (Palacios et al., 2004). *btz* mutants display diffuse *osk* mRNA in the oocyte. In *btz* mutants, the *osk* message does not colocalize with microtubules and the microtubule motor proteins, Dynein heavy chain (Dhc) and Kinesin heavy chain (Khc), when visualized together using in situ hybridization and cryoimmuno-electron microscopy (ISH-EM) (Trucco et al., 2009). This suggests the *osk* localization defects of the various EJC mutants are due to the EJC being required for proper association with microtubule motor proteins. The EJC is deposited 5' of exon-exon junctions concomitant with splicing, and *osk* contains three introns. Proper splicing of the *osk* mRNA has been shown to be required for proper localization and translation of the message, with splicing at the first intron being essential for proper localization (Hachet and Ephrussi, 2004). Taken together, this suggests that the EJC is required for the proper localization of *osk* mRNA and is loaded onto the RNA in a manner dependent on splicing at the first intron.

In addition to the EJC, Hrp48 associates with *osk* in the nucleus and has been shown to be required for association with microtubule motor proteins (Mhlanga et al., 2009; Trucco et al., 2009). Hrp48 binds to the 5' portion of the *osk* message as well as

the AB and C regions within *osk*'s 3'UTR and co-localizes with *osk* throughout oogenesis (Gunkel et al., 1998; Huynh et al., 2004; Yano et al., 2004). Proper *osk* localization and translational regulation is disrupted in *hrp48* mutants (Huynh et al., 2004; Yano et al., 2004). ISH-EM data of *hrp48* mutant egg chambers show that *osk* mRNA does not localize with microtubules, Dhc, and Khc (Trucco et al., 2009). Therefore, the disruption of *osk* RNP localization in *hrp48* mutants may be due to Hrp48 being required for the association of the *osk* RNP with microtubule motor proteins.

Staufen is also a protein required for proper localization of the *osk* message. Staufen has 5 double stranded RNA binding domains (dsRBDs) and colocalizes with *osk* mRNA in the oocyte (St Johnston et al., 1991; St Johnston et al., 1992; Micklem et al., 2000; Trucco et al., 2009). Direct interaction between *osk* and Staufen has not been demonstrated, but in Staufen mutants, *osk* is not properly localized to the posterior (Ephrussi et al., 1991; Kim-Ha et al., 1991) and as such does not produce Osk protein (Kim-Ha et al., 1995, Markussen et al., 1995, Rongo et al., 1995). In addition, Staufen transport to the oocyte is dependent on *osk* RNA, as Staufen is not localized to the oocyte in an *osk* RNA null background (Jenny et al., 2006). Therefore, the *osk* transcript and Staufen are linked in the egg chambers even though a direct interaction has not been demonstrated. ISH-EM data from *staufen* mutants show that *osk* mRNA colocalization with microtubules, Dhc, and Khc is reduced as compared to wild type egg chambers, indicating Staufen may recruit or stabilize microtubule motor proteins onto the *osk* RNP (Trucco et al., 2009).

osk mRNA's final destination is the posterior cortex of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). The oocyte cortex is where the microtubule network and filamentous actin converge, and this interaction has been implicated in the proper anchoring of the *osk* message at the posterior cortex of the oocyte. Myosin-V is an actin

dependent motor protein, and Myosin-V mutants localize Staufen protein to the posterior cortex as well as to a dot in the middle of the oocyte at stage 9, which indicates that a portion of *osk* mRNAs are mislocalized (Krauss et al., 2009). This is supported by *osk* mRNA and Osk protein failing to be tightly localized to the oocyte posterior in the Myosin-V mutant background. A Myosin-V-GFP fusion protein co-immunoprecipitates with *osk* mRNA suggesting Myosin-V and *osk* physically interact. Myosin-V has been shown to interact with Khc in a yeast-two-hybrid assay, and the two proteins seem to act antagonistically to each other from genetic studies using a Myosin-V mutant allele, $didum^{88}$, and a *khc* mutant allele (Krauss et al., 2009). This suggests that when the *osk* transcript reaches the posterior cortex of the oocyte, microtubule motor proteins and actin motor proteins act in concert to ensure the *osk* mRNA is anchored to the oocyte posterior.

OSK TRANSLATIONAL REPRESSION DURING TRANSPORT

As *osk* is transported to the posterior, it needs to be translationally repressed since ectopic expression of Osk protein leads to patterning defects in the embryo (Kim-Ha et al., 1995). The first protein shown to bind and translationally repress *osk* mRNA was Bruno (Bru), a nuclear shuttling protein that may first bind to *osk* in the nurse cell nuclei (Kim-Ha et al., 1995; Snee et al., 2008). Bru binds to sites called Bruno Response Elements (BREs) in the AB and C regions within *osk*'s 3'UTR. When the BREs are mutated, Bru's binding to the AB and C regions is greatly reduced in a UV crosslinking assay. Mutation of the BREs in an *osk* transgene also leads to precocious expression of *osk* and bicaudal embryos (Kim-Ha et al., 1995), indicating that Bru's binding to RNA is required for its ability to translationally regulate RNA. There are two models to explain how Bru regulates Osk translation. The first model has Bru binding to an eIF4E binding

protein, Cup, with Cup blocking initiation of translation (Nakamura et al., 2004). The second model postulates that Bru forms an RNP with *osk* that prevents the translational machinery from interacting with *osk* mRNA, therefore blocking translational initiation (Chekulaeva et al., 2006).

The first model comes from data showing Bru interacts with Cup, which in turn interacts with the cap binding protein, eIF4E (Wilhelm et al. 2003; Nakamura et al., 2004; Zappavigna et al., 2004). *cup* mutants translate Osk prematurely during oogenesis but do not alter *osk* localization, suggesting *cup* is only involved in translational regulation of *osk* but not required for proper localization (Wilhelm et al. 2003; Nakamura et al., 2004). Cup competes with eIF4G to bind to eIF4E, which has led to the model that the Bru-Cup-eIF4E interaction blocks initiation of translation by blocking eIF4E-eIF4G interaction (Wilhelm et al. 2003; Nakamura et al., 2004; Zappavigna et al., 2004).

The second model for Bru mediated repression is that Bru could function to aid in the formation of silencing particles, which have been defined as an RNA-protein complex that blocks ribosomes from loading onto transcripts thus blocking translation (Chekulaeva et al., 2006). Two AB regions were placed within the 3'UTR of a luciferase reporter to study translation, and it was shown that the AB regions repressed translation independent of the 5' cap, which agrees with a previous study using the *osk* 3'UTR in a similar in vitro translation assay (Lie and Macdonald, 1999; Chekulaeva et al., 2006). A potential mechanism of cap independent repression could be the formation of an RNA-protein aggregate that blocks ribosomal interaction with mRNA. To study the formation of RNA-protein aggregates, sucrose density gradient centrifugation was used. The presence of the two AB regions within an RNA induces the formation of a large particle that blocks the RNA's association with the 48S and the 80S ribosomal subunits (Chekulaeva et al., 2006). This suggests the large particles are blocking the ribosomal

subunits' association with the RNA independent of Cup through the formation of an RNP termed a silencing particle. The concern about the significance of these results is that the RNA used for the sucrose density gradient centrifugation doesn't resemble the native *osk* transcript. The RNA used in the centrifugation is made up almost entirely of Bru binding regions, while the Bru binding regions in the *osk* mRNA make up about ten percent of the total transcript. Therefore, RNA used in the centrifugation could facilitate the formation of a Bru RNP due to the fact that the RNA is almost entirely composed of Bru binding regions.

Evidence that *osk* mRNA is co-packaged in large RNPs in vivo is the phenomenon of RNA piggybacking. An RNA, containing the *osk* 3'UTR but not the *osk* coding region, localizes to the oocyte posterior in an Osk protein null background, but the same RNA does not localize to the posterior in an *osk* RNA null background (Hachet and Ephrussi, 2004). This suggests the RNA containing only the *osk* 3'UTR is localized to the posterior through a linkage to endogenous *osk* mRNA, either by direct interaction or an interaction mediated by a protein. The silencing particle model implicates Bru as the protein that mediates the linkage between the RNAs (Chekulaeva et al., 2006). However, BREs are neither required nor sufficient for RNA piggybacking of *osk* 3'UTR containing RNAs in vivo (Besse et al., 2009). This suggests a protein other than Bru may be required for the formation of silencing particles or that silencing particles are an artifact of the assay.

Current evidence points to Poly-pyrimidine Tract Binding Protein (PTB) as the protein required for piggybacking of *osk* 3'UTR containing RNAs. PTB colocalizes with *osk* mRNA throughout oogenesis and binds *osk*'s 5' region as well as *osk*'s 3'UTR. Piggybacking of *osk* 3'UTR containing RNAs to endogenous *osk* mRNA is abrogated in *hephaestus* (*heph*) mutants. *heph* is the gene that encodes PTB, suggesting PTB is

required for piggybacking. *heph* mutant oocytes display ectopic Osk accumulation as well. Ectopic accumulation occurs as early as stage 5, and by stage 7, 30% of $heph^{1545}$ mutant oocytes and 50% of $heph^{03429}$ mutant oocytes display ectopic Osk accumulation. Together, the data implicate PTB in *osk* translational repression (Besse et al., 2008), perhaps through the formation of higher-order *osk* RNPs that limit the availability of *osk* mRNA to translational machinery.

OSK TRANSLATIONAL ACTIVATION UPON LOCALIZATION TO THE OOCYTE POSTERIOR

A still unresolved question is how is translation of the Osk protein activated once the *osk* transcript reaches the oocyte posterior in late stage egg chambers. There are two potential mechanisms for how this might be achieved: (1) the recruitment of a protein to the RNA to promote activation of translation; (2) relief from repression by the removal or inactivation of the translational repressor proteins bound to the RNA, or both. A candidate protein for promoting activation of Osk translation is Vasa. Vasa colocalizes with *osk* mRNA at the oocyte posterior from stage 9 of oogenesis (Liang et al., 1994), and there is a reduction in Osk protein in *vasa* mutants (Rongo et al., 1995). Vasa is an ATP dependent DEAD Box helicase (Liang et al., 1994) and could achieve *osk* translational activation by removal of microRNAs (miRNAs). miRNAs are small RNAs of about 22nt in length that interact with mRNA to block translation (Bartel, 2004), and miRNA mediated translational repression has been shown to be active during oogenesis (Reich et al., 2009). Vasa may activate translation by restructuring the RNA to enhance translation through Vasa's helicase activity. The precedent for this comes from another DEAD Box helicase, eukaryotic initiation factor 4A (eIF4A). eIF4A melts secondary structures in the mRNA 5'UTR, which aids ribosome scanning (Gingras et al., 1999).

Recruitment of Vasa to the *osk* transcript could be achieved one of two ways. Vasa could be recruited to *osk* mRNA through the interaction with a protein already bound to the RNA, or Vasa could bind the *osk* message directly. For the first possibility, Vasa could be brought to the *osk* transcript via Vasa's interaction with Bru (Webster et al., 1997). Evidence for the second possibility comes from Vasa's interaction with a U-rich motif in *mei-P26*'s 3'UTR (Liu et al., 2009). Vasa could be recruited to *osk* by a similar U-rich motif present in *osk*'s 3'UTR.

Activation of Osk translation may also be achieved by the inactivation of Bru and Cup mediated translational repression from the transcript. A kinase localized to the posterior of the oocyte could phosphorylate Bru and/or Cup to remove the proteins' influence from the RNA and allow initiation of translation. Examples of this method of translational activation are present in yeast and dendrites (Besse and Ephrussi, 2008). In yeast, ASH1 RNA is localized to the cortex of budding daughter cells where Ash1p is then translated (Long et al., 1997; Gonzalez et al., 1999). Khd1 and Puf6 proteins block initiation of translation, and when the ASH1 transcript is localized to the cortex, both proteins are phosphorylated by membrane bound kinases, which disrupts the proteins' interaction with ASH1 mRNA and allows for translational initiation of Ash1p (Paquin et al., 2007; Deng et al., 2008).

A *Drosophila* kinase that could phosphorylate Bru or Cup is Protein Kinase A (PKA). An overactive mutant of PKA displays a bicaudal phenotype and ectopic localization of Osk protein at the oocyte anterior and in the nurse cells (Yoshida et al., 2004). This could be due to ectopic translation of Osk in these regions. To determine if reduced PKA activity leads to a reduction in Osk translation, the inhibitory subunit of the PKA heterotetramer was overexpressed, and the amount of Osk protein produced in the overexpression background was compared to the wildtype background. The amount of

Osk protein in the overexpression background was reduced in comparison to the wild type background. This suggests that PKA is required for activation of Osk translation (Yoshida et al., 2004).

Vasa and PKA have not been shown to directly bind to the *osk* RNA, so it is unknown if there are cis-acting elements that the proteins bind to regulate Osk translational activation. Activation has been shown to be regulated by cis-acting elements within the *osk* mRNA however. One of these activating cis-acting elements was identified through an in vitro selection assay using the protein Imp as the selective protein. Imp has been shown to directly bind to *osk* mRNA and colocalizes with *osk* at the oocyte posterior in late stage egg chambers (Munro et al., 2006). Imp contains 4 KH domains, and the third KH domain was used for an in vitro selection with an initially random pool of RNA. The predominant sequence motif from the selection, UUUAY, is also present in the *osk* 3'UTR. This consensus sequence was called an Imp Binding Element (IBE), and there are 13 IBEs in the *osk* 3'UTR (Munro et al., 2006). Mutating the IBEs abolishes *osk* mRNA translational activation as well as eliminating Imp localization at the posterior of stage 9 and 10 oocytes. This suggests Imp's posterior localization is due to its interaction with the *osk* message and that Imp is required for *osk* translational activation (Munro et al., 2006). However, Imp null mutants have a wild type phenotype, suggesting that an additional protein also binds the IBEs and may act, possibly redundantly with Imp, to activate Osk translation.

A translational activating cis-acting element is also present in the *osk* coding region between the first and second start codons (Gunkel et al., 1998). A *lacZ* reporter was constructed with the 5' portion of the *osk* transcript in frame with the *lacZ* coding region and under the control of the *osk* 3'UTR. The *lacZ* reporter in a wild type background was translated in the same fashion as the native *osk* transcript. The lacZ

mRNA was localized to the oocyte posterior where LacZ protein translation was initiated in stage 9 oocytes (Gunkel et al., 1998). When the portion of the transcript containing the activating element was deleted, translation of the LacZ protein was lost. When this transcript also contained a mutation in one of the BREs, translation of the LacZ protein was restored, suggesting that the 5' activation element works as a derepressor of Bru mediated translational regulation (Gunkel et al., 1998). Hrp48 has been shown to bind to both the region containing the 5' activation element and the AB region. No further work was done on these findings, so it is unknown if Hrp48 or some other protein such as PTB links the 5' region with the 3' region to regulate translation.

A long poly(A) tail on mRNA has also been shown to be required for proper translation of a transcript through multiple mechanisms. The poly(A) tail can aid export from the nucleus, stabilize the transcript, and enhance translation (Moore and Proudfoot, 2009). The protein Orb is required for long a poly(A) tail length on the *osk* mRNA (Chang et al., 1999; Castagnetti and Ephrussi, 2003). Orb protein interacts with *osk* mRNA in a UV crosslinking assay (Chang et al., 1999). Orb also co-localizes with *osk* mRNA in the oocyte during early stages of oogenesis and at the oocyte posterior in stage 8 – 10 egg chambers (Christerson and McKearin, 1994). This interaction may be important for Osk translational activation since weak *orb* mutants display a reduced amount of Osk protein at the oocyte posterior (Chang et al., 1999). Orb's interaction with *osk* may not be required for Osk translational activation. However, since *orb* mutants also fail to localize *osk* mRNA to the oocyte posterior (Christerson and McKearin, 1994), the localization failure is likely due to an indirect effect on microtubule organization. *orb* mutants have been shown to disrupt microtubule organization in stage 9 oocytes as well as display premature cytoplasmic streaming at stage 9 (Martin et al., 2003).

Translational activation of the *osk* transcript is a complex process that involves many seemingly unrelated factors, and the contributions of these factors are still not fully defined. Vasa is known to be required for translation, but it is unknown how Vasa contributes to activation. Orb is a protein required for activation by enhancing translation through maintenance of the poly(A) tail. Although, there is evidence that Orb is involved in localization of transcripts (Christerson and McKearin, 1994; Chang et al., 1999; Martin et al., 2003). Cis-acting elements have also been identified that are required for proper *osk* translation, but trans-acting factors that may interact with the cis-acting elements have yet to be identified. In the case of the IBEs, they were identified through their interaction with Imp, but Imp mutants do not display the same phenotypes as the *osk* IBE mutants (Munro et al., 2006). Activation of localized *osk* could be achieved through the phosphorylation of a repressor protein such as Bru. So far, PKA is the only good candidate for the kinase that could phosphorylate Bru.

SUMMARY AND CONCLUSIONS

The processing, transport, and localized translation of the *osk* message at the posterior of stage 9 oocytes is no small feat. Multiple proteins interact with *osk* mRNA throughout its existence and transport to ensure that Osk protein is translated at a specific time and place. These proteins or protein complexes often have multiple roles in the life cycle of the *osk* message. *osk* forms a ribonucleoprotein (RNP) in the nurse cell nuclei, and the *osk* RNP is a dynamic particle that interacts with different subsets of proteins as it passes through the various regions of the egg chamber. These proteins share two common goals, get *osk* to where it needs to be and make sure *osk* begins translation at the correct time and place. The proteins accomplish these goals through various different

mechanisms, whether it be through stabilization of the RNA, through direct inhibition of translation, aiding interaction with the microtubule motor proteins, or through enhancing Osk protein stability through post translational modification.

OVERVIEW OF THE DISSERTATION RESEARCH

There are two main aims of the research in this thesis. The first is to determine the sequence of the Bru binding sites using an in vitro selection assay, and the second is to determine if the sites found from the in vitro selections regulate translation in vivo.

To determine the sequence of the Bru binding site, I carried out an in vitro selection using a pool of RNAs containing a random 50nt region within an 110nt fragment of RNA. I used the full-length Bru protein as well as two Bru fragments that bind with the same specificity as the full-length Bru protein for the selection. The first fragment contained the first two RRMs of Bru and was termed RRM1+2. The second fragment contained the C-terminal RRM plus 42 flanking amino acids, and this fragment was termed RRM3+. None of the selections produced RNAs with overrepresented secondary structures in the final round pools of RNA. The RRM3+ final round pool contained three primary sequences that were overrepresented, CAAAGUNUUCYR, UUAUAUG, and UGCAGU. The RRM1+2 final round pool did not contain overrepresented, long sequence motifs. Therefore, the frequency of tetranucleotide sequences was analysed. Several overrepresented tetratnucleotide sequences were identified and categorized into two subsets, a subset consisting of only U and purines and a subset enriched in C and A. The full-length Bru final round pool contained one overrepresented sequence, UUGUCY. In addition, the full-length Bru final round pool of RNAs also contained examples of the RRM3+ sequence motifs near the RRM1+2 motifs,

which were termed combinatorial sites. At least one of the motifs from the three selections was present in each of the native targets of Bru, with some of the Bru regulated mRNAs containing more than one motif.

Two methods were used to determine if the overrepresented sequences were biologically relevant. The first method entailed mutating the sites in the *osk* 3'UTR. The *osk* 3'UTR contains three classes of potential Bru binding sites in the AB and C regions. These sites were mutated in an *osk* genomic construct and introduced into an *osk* RNA null and an Osk protein null background. Embryos from mothers carrying these genotypes were assayed for phenotypic effects in Osk activity. The amount of Osk protein at the posterior of late stage oocytes and early stage embryos was also assayed. Mutation of all of the sites in the AB region had no phenotypic effects. Mutation of the sites in both the AB and C region resulted in defective translational repression of the Osk protein in the *osk* RNA null background. Mutating the sites in the C region led to a reduction in Osk translation and Osk activity in the *osk* RNA null background. This suggests the AB and C regions act together to achieve translational repression, while the C region has an additional role in activation. Unexpectedly, the phenotypes were rescued in the Osk protein null background, indicating that *osk* mRNA regulates translation of other *osk* transcripts in trans.

The second method involved a GFP reporter transgene containing a 100nt fragment of the SV40 3'UTR with four copies of the different selected sequence motifs spaced 20nt apart embedded in the SV40 fragment. Six different motifs were used for the assay as well as a negative control that contained no motifs in the SV40 fragment. When the GFP transgenes were introduced into a wild type background, all of the motifs displayed reduced amounts of GFP in the egg chambers in comparison to the negative control. Bru is encoded by the *arrest* (*aret*) gene. When the transgenes were introduced

into an *aret* mutant background, the amount of GFP in the egg chambers was greater in the *aret* mutant background in comparison to an *aret* heterozygous background except for the negative control. The negative control transgene contained nearly an equal amount of GFP between the two backgrounds. Therefore, the selected motifs repressed the translation of the GFP transgenic RNA, and the repression was dependent on the presence of Bru.

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Chapter 2: Multiple RNA binding domains of Bru confer recognition of diverse binding sites for translational repression

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ABSTRACT

Bruno protein binds to multiple sites – BREs – in two regulatory regions of the *oskar* mRNA 3' UTR, thereby controlling *oskar* mRNA translation. Bruno also binds and regulates other mRNAs, although the binding sites have not yet been defined. Bruno has three RRM type RNA binding motifs, two near the amino terminus and an extended RRM at the C terminus. Two domains of Bruno - the first two RRMs (RRM1+2), and the extended RRM (RRM3+) - can each bind with specificity to the *oskar* mRNA regulatory regions. In vitro selections with Bruno and with each of the RNA binding domains revealed complexity in Bruno RNA binding specificity. The anti-RRM3+ aptamers include long, highly constrained motifs, including one corresponding to the previously identified BRE. The anti-RRM1+2 aptamers lack constrained motifs, but are biased towards classes of short and variable sequences. Bruno itself selects for several motifs: some are those bound by RRM3+, but the predominant motif is distinct and suggests the existence of a third RNA binding domain. We propose that the multiple RNA binding domains allow combinatorial binding in which extended Bruno binding sites are assembled from sequences bound by the individual domains, with RRM3+ being the primary determinant of specificity. Examples of several types of motifs were identified in known targets of Bruno, and tested for function using transgenic reporter mRNAs. When present in multiple copies, most motifs conferred Bruno-dependent translational repression. These results suggest that other proteins with multiple RRMs may employ combinatorial binding to achieve high levels of specificity and affinity.

INTRODUCTION

Recognition of RNA sequences or structures by proteins is crucial for many aspects of RNA function. For gene expression, proteins bind to mRNAs in the nucleus to mediate their processing, splicing and nucleocytoplasmic transport. In the cytoplasm RNA binding proteins are required for translation, and contribute to a wide range of post-transcriptional control events including subcellular localization, translational repression and activation, and mRNA decay and stabilization. The selective nature of these regulatory events – not all mRNAs are treated the same – demands a means for the specific recognition of individual mRNAs. Each such mRNA contains appropriate cis-acting regulatory elements, and RNA binding proteins bind with specificity to the elements (Dreyfuss et al. 2002).

RNA binding is mediated by a variety of protein domains (Chen and Varani 2005; Lunde et al. 2007). The most common is the RNA recognition motif or RRM. This domain is found in many proteins, with about 2% of human proteins containing one or more RRMs (Varani and Nagai 1998; Lunde et al. 2007). RRM domains typically bind single stranded RNA, although there are examples of binding to single stranded DNA or proteins. The RRM consists of four anti-parallel beta strands and two alpha helices, arranged in an alpha/beta sandwich. Contacts with RNA occur along the beta sheet, with side chains stacking with the bases. Interactions between the core RRM and RNA typically span up to four nucleotides, providing only limited specificity in binding (Maris et al. 2005). Many RRMs rely on additional structural features to expand the RNA binding surface and increase specificity and affinity. These additional features vary considerably among different proteins, and involve additions to the N- or C-termini or expansion of loops connecting the secondary structure elements (Cléry et al. 2008).

Often, proteins contain two of more copies of the RRM. Structural studies have revealed different options for the contributions of tandem RRMs to RNA binding. In each case the use of two RRMs increases the size of the RNA sequence recognized, with two themes for how this is accomplished. In the first, the two RRMs interact with a single region of RNA. Tandem RRMs of the Sex lethal and HuD proteins create a cleft in which the RNA lies, the RRMs of nucleolin form a sandwich with the RNA in the middle, and two RRMs of the poly(A) binding protein (PABP) form an extended binding platform (Handa et al. 1999; Wang and Tanaka Hall 2001; Allain et al. 2000; Deo et al. 1999). Alternatively, tandem RRMs bind to sequences separated from one another in the mRNA. This option is displayed by the two carboxyl terminal RRMs (RRMs 3 and 4) of polypyrimidine tract binding protein (PTB). Interactions between the two RRMs position the RNA binding surfaces apart from one another, and the RNA substrate must contain a spacer between its binding sites for high affinity binding (Oberstrass et al. 2005).

Bruno (Bru) is an RRM protein from *D. melanogaster* (Webster et al. 1997). Bru binds to a number of different mRNAs in the ovary and regulates their translation (Kim-Ha et al. 1995; Filardo and Ephrussi 2003; Yan and Macdonald 2004; Sugimura and Lilly 2006; Wang and Lin 2007; Moore et al. 2009). Regulation by Bru is best characterized for the *oskar* (*osk*) mRNA. Bru binds to two different portions of the *osk* mRNA 3' UTR, the AB and C regions. Sequence alignment and mutagenesis studies identified Bru binding sites, BREs (Bru Response Elements), within these regions. Mutation of the BREs greatly reduces Bru binding in vitro and disrupts translational regulation in vivo (Kim-Ha et al. 1995). The *osk* AB and C region BREs mediate translational repression, and the *osk* C region BREs have an additional role in translational activation (Kim-Ha et al. 1995; Reveal et al. 2010). The role of the BREs in translational activation may well

require other proteins that bind close to the BREs, and it seems likely that repression is the default role of Bru binding sites (Reveal et al. 2010).

Bru contains three RRMs, organized in a manner shared by several families of RRM proteins: there are two tandem RRMs near the amino terminus of the protein, and a third RRM positioned at the C terminus (Webster et al. 1997). None of the isolated RRMs of Bru individually displays RNA binding, but two larger domains of Bru each bind to the *osk* AB and C regions. One domain (RRM1+2) consists of RRMs 1 and 2, and the other (RRM3+) consists of RRM3 plus the final 42 amino acids of the spacer linking RRM2 and RRM3 (Fig. 2.1). Both domains show specific binding to the *osk* regulatory regions, but binding of the RRM3+ protein is more sensitive to mutation of the BREs and thus has a higher degree of specificity for the BREs (Snee et al. 2008; Lyon et al. 2009). Although Bru binds in vitro to the 3' UTRs of other regulatory targets, including the *gurken* (*grk*), *cyclin* A (*cycA*), *Sex lethal* (*Sxl*) and *germ cell-less* (*gcl*) mRNAs, only the *cycA* 3' UTR has a perfect match to the consensus BRE sequence (Sugimura and Lilly 2006; Filardo and Ephrussi 2003; Yan and Macdonald 2004; Wang and Lin 2007; Moore et al. 2009). Therefore, it seems clear that the BRE consensus sequence provides an incomplete picture of the Bru binding site or sites.

To better characterize Bru binding sites, and to begin to understand how the different RNA binding domains of Bru contribute to specificity of binding, we used in vitro selection methods to identify preferred binding sequences for Bru and for the isolated RNA binding domains of Bru. The results indicate that the RRM3+ domain is the major determinant of binding specificity, and that Bru may bind to extended sites consisting of both the highly constrained RRM3+ binding motifs and nearby sequences showing a preference for RRM1+2 binding. From the results of the selections, candidate

Bru binding sites were identified in the *grk* and *cycA* 3' UTRs. These sites conferred Bru-dependent translational repression on reporter mRNAs in the ovary.

RESULTS

In vitro selection of aptamers

To obtain a more complete understanding of Bru RNA binding specificity we performed in vitro selection of binding sites [SELEX; (Tuerk and Gold 1990)] with Bru proteins. The proteins used for this analysis were full length Bru, as well as the Bru RRM1+2 and RRM3+ domains. Although the known Bru binding sites define a short consensus sequence (7-9 nt), the RNA for the selections included a longer random sequence (50 nt) to ensure that larger sequence motifs, or motifs that must be presented within larger structures, could be bound and recovered. Progress of the selections was monitored by testing naive and selected RNA pools for binding. Each selection led to an increase in affinity, with larger increases for the RRM3+ and Bru selections (Fig. 2.8). After 11 rounds of selection, bound RNAs from the final round were converted to DNA and sequenced. Each family of aptamers (Tables 2.3-2.5) was evaluated for overrepresented primary sequences using pattern searches and MEME (Bailey and Elkan 1994), and for secondary structures using MFOLD (Zuker 2003; Mathews et al. 1999). The highest degree of specificity was found for RRM3+ and Bru, and these results are presented first.

The selection against RRM3+ identified a relatively long sequence motif, 5' CAAAGUNUUCYR (Y, pyrimidine; R, purine). From the 32 sequenced clones of the final round of selection, 16 had the consensus sequence or a close variant (Fig. 2.2A). The appearance of such a long sequence motif was surprising: the core RRM domain

typically binds to three nucleotides, and RRMs with structural features that extend the core typically recognize sequences no longer than 6 or 7 nucleotides (Chen and Varani 2005; Auweter et al. 2006). The RRM3+ domain has an additional structural element that is important for binding (Lyon et al. 2009), but recognition of a 12 nucleotide sequence by a single RRM is unprecedented. One plausible explanation is that a portion of the sequence is required indirectly for correct presentation of the actual binding site, as is the case for recognition of U1 snRNA by the N terminal RRM of the U1A protein (Oubridge et al. 1994). However, there is no structure predicted to be formed by the isolated CAAAGUNUUCYR sequence, or by the sequence in the context of the anti-RRM3+ aptamers. Notably, this sequence did not appear among the anti-Bru aptamers (below). Therefore, it is possible that recognition of CAAAGUNUUCYR relies in part or whole on an unconventional form of RNA binding by RRM3+, perhaps relying on surfaces normally not accessible or folded differently in the context of Bru.

Two other motifs identified among anti-RRM3+ aptamers by MEME analysis are UGCAGU and UUAUAUG. The UGCAGU motif appeared 4 times, with 18 additional copies with a single mismatch (Fig. 2.2B). The UUAUAUG motif appeared 6 times, with 3 additional copies with a single mismatch (Fig. 2.2C). A notable feature of the UUAUAUG motif is that it matches almost fully with the previously defined BRE consensus sequence [U(G/A)U(A/G)U(G/A)U].

From the selection for RNAs that bind to full length Bru, motifs related or identical to those identified in the anti-RRM3+ aptamers might have been expected to predominate. However, the predominant motif from the MEME analysis is UUGUCY. In the 90 clones sequenced, there are 92 instances of UUGUCY and 173 additional copies with a single mismatch. This motif is not obviously related to any of the RRM3+ motifs. Two of the motifs identified in the selection with RRM3+ do appear in the anti-Bru

aptamers, but at lower frequencies than UUGUCY. There are many copies of the BRE-like UUAUAUG motif: 3 are perfect copies and an additional 21 have a single mismatch. There are no perfect matches to the UGCAGU motif, but there are 6 examples with a single mismatch. There are no close matches to the longer CAAAGUNUUCYR RRM3+ motif.

In contrast to the results with RRM3+ and Bru, selections against RRM1+2 did not produce highly overrepresented sequence motifs detected by MEME analysis. Following the approach taken by (Faustino and Cooper 2005) in their analysis of aptamers selected by the related mouse Etr-3 protein (also known as BrunoL3), the frequencies of tri- and tetra-nucleotides were determined, and pattern searches tested whether the most abundant tetranucleotides were core sequences of longer motifs. No single long and highly overrepresented motif was discovered. Instead, we identified a number of frequently appearing tetranucleotides (Table 2.1 & 2.6).

One subset of the tetranucleotides consists only of U and purines (U/R-rich); some of these form the core of the most common pentanucleotide, UUAUG, which appears 19 times in the aptamers. This pentanucleotide can be superimposed on the 5' portion of the BRE consensus sequence, which is U(G/A)U(A/G)U(G/A)U. The U/R-rich tetranucleotides appear frequently within the AB and C regions of the *osk* 3' UTR, the two regions implicated in Bru-mediated translational control (Table 2.1), suggesting that enrichment of these short sequences in the anti-RRM1+2 aptamers may have biological relevance.

Another subset of tetranucleotides are enriched in C and A (at least 3 of the 4 nucleotides); some of these form the core of the second most common pentanucleotide, UCAAA, which appears 18 times. The C/A-rich tetranucleotides are not closely related to

the BREs, and are almost completely absent from the *osk* 3' UTR AB and C regions (Table 2.1). Thus, there is no prediction about their biological relevance.

For comparison of anti-RRM1+2 and anti-Bru aptamers, the frequencies of tetranucleotides were also determined in the anti-Bru aptamers (Table 2.6). Not surprisingly, tetranucleotides from the predominant Bru motif (UUGUCY) are among the most highly represented. In addition, four of the six most common tetranucleotides contain 3 U's and one G, a trend similar to the enrichment of U/R-rich tetranucleotides in the anti-RRM1+2 aptamers.

The absence of one or even a few dramatically overrepresented sequence motifs in the anti-RRM1+2 aptamers suggests that the specificity of RRM1+2 is low. There is some degree of specificity, since binding of RRM1+2 to the *osk* AB region RNA is diminished by mutation of the BREs (Snee et al. 2008). Consistent with at least weak specificity, the pool of RNAs selected by RRM1+2 binds better than the naive pool (Fig. 2.8). The specificity for the *osk* AB region may well involve binding to the many U/purine rich sequences in that region, some of which are altered by mutation of the BREs (Kim-Ha et al. 1995).

While the enrichment of particular sequence motifs is one outcome of the selections, there were also changes in nucleotide composition which were most striking for the anti-Bru aptamers. The initial template pool of random DNA sequences was synthesized with equal amounts of each nucleotide. Following selection with Bru, the residue U was enriched from 25% to 50% while the residues A and G were reduced in the population to less than 15% each (Table 2.2). This level of U frequency is similar to that of the *osk* AB region, which has the strongest Bru binding of the two *osk* regulatory regions (Kim-Ha et al. 1995). The anti-Bru aptamers differ from the *osk* AB region for frequency of C residues: the *osk* AB region is only 10% C, but C is the second most

abundant nucleotide (23%) in the anti-Bru aptamers. Less extreme changes in nucleotide composition occurred in the other selections. For both anti-RRM1+2 and anti-RRM3+ aptamers the frequency of U increased slightly, but not to the same extent as for the anti-Bru aptamers. Unlike the anti-Bru aptamers, there was no reduction in the frequency of A in the anti-RRM1+2 and anti-RRM3+ aptamers (Table 2.2).

The results of the selections reveal a complex picture of Bru binding specificity, with evidence for three binding domains, each of which displays different types of specificity. The RRM3+ domain appears to provide a high degree of binding specificity, as highly constrained motifs of 6 nt or longer were identified. A binding domain or activity only found in full length Bru also has a high degree of specificity, recognizing a highly constrained 6 nt motif. This binding activity could rely on one or both of the RRM binding domains but with a different specificity imposed by the organization or folding of the domains in the context of Bru, or it could rely on a separate RNA binding domain. By contrast, the RRM1+2 domain does not have a high degree of specificity but may bind preferentially to regions enriched in certain types of sequences. Since anti-Bru aptamers contain both the well defined RRM3+ motifs and at least some of the shorter sequences identified from the RRM1+2 selection, it appears that Bru may recognize combinatorial sites in which the different domains of Bru bind independently to different motifs (see Discussion).

Candidate Bru binding motifs in known targets of Bru regulation

Five genes have been reported to be subject to regulation by Bru. The best characterized example of such regulation is *osk*, which contains BREs in the AB and C regions of its 3' UTR. Mutation of the BREs substantially reduces Bru binding in vitro,

and leads to translational defects in vivo (Kim-Ha et al. 1995; Reveal et al. 2010). The BREs closely resemble the UUAUAUG motif selected by RRM3+. Other features identified by the selections are also found in the AB and C regions. Both regions are highly enriched in short U/purine sequences, as found in the anti-RRM1+2 and anti-Bru aptamers (Table 2.1). Such sequences are contained within the BREs and within the UUAUAUG motif, which may explain their prevalence in the *osk* regulatory regions. In addition, the *osk* AB and C regions are also substantially enriched in short U/G sequences, which can overlap with the BREs and UUAUAUG motif, but do not correspond precisely to those motifs. Perfect copies of the predominant UUGUCY Bru motif are present once in the AB region and twice in the C region. Finally, a sequence that differs at only one position relative to the CAAAGUNUUCYR motif selected by RRM3+ is present in the C region. Based on the results obtained here we mutated the UUGUCY and CAAAGUNUUCYR-like motifs within the *osk* mRNA (Reveal et al. 2010). Mutation of either the single UUGUCY site in the *osk* AB region or the two UUGUCY sites in the *osk* C region did not detectably alter translational repression. This is not surprising, as the AB and C regions act redundantly in repression. However, mutation of these sites in the *osk* C region did disrupt translational activation, just as for mutation of the *osk* C region BREs. Similarly, mutating the single CAAAGUNUUCYR-like motif affected only translational activation, although less severely than mutation of either the BREs or the UUGUCY sites. The weaker effect is not surprising, as mutation of the CAAAGUNUUCYR-like site has only a very modest effect on Bru binding to the *osk* C region (Reveal et al. 2010). Thus, both types of newly identified Bru binding sites can mediate translational activation, but their ability to confer translational repression has not been determined. The UUGUCY and CAAAGUNUUCYR-like sites are now called Bru type II and type III sites, respectively (Reveal et al. 2010).

The other known targets of Bru translational repression are the *cyclinA* (*cycA*), *gurken* (*grk*), *Sex lethal* (*Sxl*) and *germ cell-less* (*gcl*) mRNAs (Sugimura and Lilly 2006; Filardo and Ephrussi 2003; Yan and Macdonald 2004; Wang and Lin 2007; Moore et al. 2009). In all cases there is evidence of Bru binding to the mRNAs 3' UTR, but specific Bru binding sites have not been experimentally defined. The 3' UTRs were searched for sequences corresponding to each of the longer motifs from the RRM3+ and Bru selections (Fig. 2.3). The only perfect matches were in the *cycA* gene, with one copy of the BRE-like UUAUAUG motif and one copy of the UGCAGU motif (both from the RRM3+ selection). In both cases, the motifs are adjacent to short sequences similar to the A/C-rich tetranucleotides identified among the anti-RRM1+2 aptamers, consistent with the notion of Bru binding to combinatorial sites (see Discussion). None of the other known targets of Bru have perfect matches to any of the identified motifs. However, there are multiple copies of one or more of the motifs when a single mismatch is allowed (Fig. 2.3).

Translational repression by Bru binding motifs

Candidate Bru regulatory sites were tested for their ability to confer translational repression on a *GFP* mRNA reporter. Reporter transcripts were expressed using the UAS/GAL4 system in the germ line cells of the ovary (Rorth 1998), where Bru is present (Webster et al. 1997). The control transgene mRNA consists of the *GFP* coding region, a portion of the SV40 3' UTR, and a portion of the *fs(2)K10* 3' UTR including the polyadenylation site. To determine if the reporter mRNAs are translationally regulated, we compared mRNA and GFP protein levels by quantitative real time PCR (qRT-PCR) and confocal microscopy, respectively. The control transgene shows strong GFP

expression throughout the germ line cells of the egg chamber (Fig. 2.4B), consistent with the absence of translational repression. Derivatives of this transgene were constructed by insertion of copies of candidate regulatory sites from the known Bru targets.

Three of the sites were from the *osk* mRNA. One site consists of a BRE and flanking U/G-rich sequence, UGUUUUAUAUGU. This site is expected to mediate translational repression, based on the effect of mutating BREs in the *osk* mRNA. A second site is the UUGUCC motif (type II Bru binding site) from the anti-Bru aptamers, which appears once in the *osk* AB region and once in the *osk* C region (an additional copy in the C region has the alternate UUGUCU sequence). The *osk* C region sites are required for translational activation (Reveal et al. 2010), but it is not known if these sites also mediate repression. The third site from *osk* is UAAAGUCUUCUA (the type III Bru binding site), which differs at only one position from consensus for the CAAAGUNUUCYR motif of the anti-RRM3+ aptamers. This site makes a minor contribution to translational activation (Reveal et al. 2010).

Three other candidate regulatory sites were from the *cycA* and *grk* 3' UTRs. The *cycA* sites are those mentioned in the previous section, consisting of an RRM3+ motif adjacent to an A/C rich region: CAAUUUUAUAUGU and UCAAUUGCAGU. Within the *grk* mRNA there are no perfect matches to any of the anti-RRM3+ or anti-Bru aptamer motifs, but all three copies of the UGCAGU RRM3+ motif with a single mismatch are positioned close to U/G- or A/C-rich sequences. The candidate regulatory site chosen for analysis is UGUUUGUAGU.

In an initial round of experiments we tested reporter transgenes bearing two copies of candidate regulatory sites, one at each end of 88 nt of SV40 sequences (the control transgene also has SV40 sequences in its 3' UTR). None of these transgenes showed a large degree of translational repression (data not shown). By contrast, a similar

GFP reporter with the *osk* AB region is dramatically repressed (Reveal et al. 2010). One key difference between the *osk* AB and candidate site reporters is the density of binding sites: the reporters tested here have two sites in ~100 nt, while the *osk* AB region is similar in size but has more Bru binding sites.

In a second round of experiments we modified the reporter transgenes to add two additional copies of the candidate Bru regulatory sites, evenly spaced within the central SV40 sequences (Fig. 2.4A). Notably, each of the reporter mRNAs with candidate Bru regulatory sites had reduced levels of GFP. For the two reporter mRNAs with the strongest repression (more than 10 fold reduction), the regulatory sites (from the *osk* and *cycA* mRNAs) consist of a BRE-like sequence flanked by a short sequence similar to the enriched short motifs from the RRM1+2 selections (Fig. 2.4C, D, I). Intermediate levels of repression were conferred by three sites. One was the type II Bru binding site (the UUGUCC motif from the Bru selection)(Fig. 2.4G, I). The others were from the *cycA* and *grk* mRNAs, and consisted of a UGCAGU motif (from the RRM3+ selection) adjacent to one of the short motifs (like those from the RRM1+2 selection). The lowest level of repression was provided by the type III Bru binding site.

Repression by each of the regulatory sites is presumably due to the action of Bru. To confirm this expectation, the level of GFP produced by each reporter was compared between flies with Bru activity (*aret*/+ heterozygotes) and flies with substantially reduced Bru activity (*aret* mutant homozygotes) (Fig. 2.5). For the control reporter mRNA there was very little difference in GFP level when Bru activity was reduced (Fig. 2.5A). However, for each of the reporters showing repression, the removal of Bru resulted in an increase in GFP (Fig. 2.5B-G). Not surprisingly, the largest increase in GFP came from the reporters that are most strongly repressed.

Binding of Bru in ovary extracts to each of the multimerized elements was tested in a UV crosslinking assay. The portion of each transgene bearing the multimerized elements was transcribed and radiolabeled in vitro, incubated with ovarian extract, UV irradiated, and the adducts displayed by denaturing electrophoresis and phosphorimaging. In this assay the RNA with the multiple copies of the combinatorial UGUUUUAUAUGU site (corresponding to a sequence from the *osk* AB region) bound most strongly (Fig. 2.6A). Binding to two other combinatorial sites and the UUGUCY site was also detected, but was weaker. The other sites did not bind detectably in this assay. To confirm that binding, where it was observed, was due to Bru, competition binding assays were performed (Fig. 2.6B). The presence of unlabeled *osk* AB RNA with known Bru binding sites greatly reduced binding, confirming that the crosslinked protein is Bru.

The absence of detectable binding of some of the RNAs was surprising, given their demonstrated effects on translation. Therefore, a quantitative filter binding assay was also used with recombinant Bru (Fig. 2.6C). The RNA with the UGUUUUAUAUGU sites shows the strongest binding, just as in the crosslinking assay, and binds Bru nearly as well as the *osk* AB RNA. Not surprisingly, this RNA mediates strong repression in vivo. The other combinatorial site with a BRE-like sequence, CAAUUUAUAUGU, also shows strong binding and strong repression. The RNA with the UGUUUGUAGU sites shows weaker binding and is less effective at translational repression. Two of the RNAs (those with UUGUCC or UAAAGUGUUCUA sites) do not bind Bru any more effectively than the SV40 control in this assay, and one RNA (with the UCAAUUGCAGU sites) does not bind detectably at all. Nevertheless, each of these RNAs confers some degree of translational repression in vivo, and the repression requires Bru (Figs. 2.4 and 2.5). The probable cause of this inconsistency is that the RNAs used for binding assays are folded into structures that completely or partially mask

the binding sites. Structural predictions strongly support this view. Notably, the RNA that fails to bind is predicted to adopt an extensively and stably base paired structure, with each copy of the binding site in a double stranded region with multiple G-C base pairs (Table 2.7). This folding would very likely prevent Bru binding in vitro as none of the sites would be in a single stranded conformation with the binding site exposed. However, when the segment of RNA is embedded within a reporter mRNA in vivo, in the presence of many other RNA binding proteins, the structure may not form or may be unstable, allowing Bru to bind and repress translation. None of the other RNAs appear to have structures that would completely mask the binding sites, but most of the weakly binding RNAs have either a subset of the sites in strongly base paired helices, or all of the sites in more weakly based paired helices. By contrast, the two RNAs that bind strongly are predicted to have structures with the binding sites in regions that are only partially base paired, with primarily A-U and G-U base pairs. The exception to this trend is the RNA with the weakly repressing UAAAGUCUUCUA sites. Three of the four sites in the RNA are not expected to be in stable helices, and thus likely available for binding. However, this is the site that was recovered in the RRM3+ selection and not from the Bru selection, and is not expected to be tightly bound by Bru in vitro.

DISCUSSION

In a previous characterization of Bru RNA binding we found that two different RNA binding domains, RRM1+2 and RRM3+, could each bind to BRE-containing regulatory regions of the *osk* mRNA 3' UTR. Mutation of the BREs reduced binding, with a greater reduction for binding by RRM3+. This suggested that the domains had some specificity for BREs, and that strong binding was achieved by contributions from

both domains. Experimental evidence supported this model: mutation of RRM2 or RRM3 by alanine substitutions (predicted to interfere with RNA binding but not folding of the RRM) reduced RNA binding in vitro and partially disrupted Bru repressive activity in vivo, while mutation of both RRMs more strongly affected both RNA binding and repressive activity (Snee et al. 2008).

The selections with the Bru RNA binding domains and full length protein provide a more complete picture of the specificity of Bru RNA binding and how it is achieved. The two known RNA binding domains do not make equal contributions to specificity. Instead, the RRM3+ domain provides a high degree of specificity through recognition of motifs with highly constrained sequences, while the RRM1+2 domain displays a preference for classes of short, lower complexity sequences. In addition, a previously unrecognized RNA binding domain, detected only in full length Bru, also recognizes a highly constrained sequence. It would not have been possible to predict the existence of the third binding domain from the Bru protein sequence, and better definition of this domain will be a future goal.

In principle, the presence of multiple RNA binding domains in Bru could serve several different purposes. First, different domains could bind combinatorially, with each domain contributing only a subset of the total contacts with an extended binding site (Fig. 2.7A). Such combinatorial binding would enhance specificity and affinity of binding, and could explain why both RRM1+2 and RRM3+ are required for optimal binding of *osk* mRNA regulatory sites in vitro and for efficient translational control of *osk* mRNA in vivo: RRM3+ would provide much of the binding specificity, while RRM1+2 with its lower specificity would contribute primarily to binding affinity. Consistent with this model, the *osk* AB and C regions contain features recognized by each of the three Bru binding domains. Our understanding of the regulatory elements in the other Bru targets is

more limited. Nevertheless, it is notable that the sequences from the *cycA* and *grk* mRNAs containing one of the highly constrained RRM3+ motifs close to an example of the short, lower complexity motifs selected by RRM1+2 can confer translational repression. We propose that the multiple RNA binding domains of Bru serve primarily in allowing combinatorial binding to extended binding sites.

Second, the different domains could serve in recognition of different substrate mRNAs, expanding the spectrum of potential regulatory targets (Fig. 2.7B). For example, the mouse HuC protein, which contains 3 RRMs, has been reported to bind to two very different binding sites via different RRMs (Abe et al. 1996). Bru does bind to multiple different mRNAs, but our evidence does not point to specialization of different binding domains for binding to different mRNAs. Furthermore, the low degree of specificity provided by RRM1+2 would not be effective in limiting Bru activity to a small proportion of all mRNAs.

A third option is suggested by the ability of Bru to oligomerize RNAs bearing tandem copies of the *osk* AB region in vitro (Chekulaeva et al. 2006): the multiple RNA binding domains could bridge different molecules of target mRNAs, forming interconnected RNA/protein particles (Fig. 2.7C). From kinetic considerations it is more likely that a single molecule of Bru would make multiple contacts with a single molecule of RNA, at least in situations in which appropriate binding sites are present and can be bound without conformational constraints. After initial binding of Bru to one RNA molecule via one domain, the other domains of Bru would most rapidly encounter any additional binding sites nearby in the same molecule. However, assembly of *osk* mRNA into particles might then allow intermolecular binding of Bru: dissociation of individual Bru RNA binding domains from their substrate might be followed by binding to a different substrate positioned nearby. Such Bru dependent oligomerization of *osk* mRNA

has thus far only been demonstrated in vitro with artificial mRNAs, and the biological relevance of this form of binding remains uncertain. Although *osk* mRNA is assembled into particles in the ovary, assembly depends on Polypyrimidine Tract Binding Protein but apparently not Bru since BREs have been reported to be neither necessary nor sufficient for *osk* mRNA interaction in vivo (Besse et al. 2009).

Multiple binding specificities of Bru RNA binding domains

A notable feature of the in vitro selections with Bru or its subdomains is the recovery of more than one type of binding motif from each selection. For the RRM1+2 domain it is possible that each of the component RRMs has its own rather limited binding specificity [although neither binds well in isolation (Snee et al. 2008)], and that these specificities are revealed by the selections. However, such an argument is not possible to explain the three very different binding motifs that were identified for RRM3+. Multiple binding specificities are not uncommon for RNA binding proteins, although different binding sites are sometimes recognized by different binding domains (Abe et al. 1996). For a single RNA binding domain, different binding specificities could be obtained through alternate conformations of the domain as observed for U2AF65 (Sickmier et al. 2006; Thickman et al. 2007), or structural reorganization upon binding as for NELF-E (Rao et al. 2008). In addition, changes in RNA structure have the potential to present a site differently to a binding protein (Banerjee et al. 2003). How the flexibility of Bru binding is achieved is not known, and will likely require structural studies with the protein or domains bound to different substrates for a complete understanding.

Achieving specificity in translational control

Translational control of *osk* mRNA is essential, with defects in repression and activation from mutation of control elements blocking proper development (Kim-Ha et al. 1995; Munro et al. 2006; Reveal et al. 2010). Although many factors are involved in this control, Bru appears to play the key role of selectively recognizing the *osk* mRNA, at least for repression. RRM containing proteins often bind with modest specificity, which is not surprising given that the core RRM interacts with only 3-4 nucleotides of an RNA. As noted above, the multiple RNA binding domains of Bru provide a means to obtain a much higher degree of specificity. However, even that level of specificity might not be sufficient to allow efficient repression of osk mRNA but not the many other mRNAs that might, fortuitously, have sequences resembling Bru binding sites. Two lines of evidence indicate that multiple Bru binding sites are required for a significant level of translational control in vivo. First, *osk* mRNA has many Bru binding sites and mutation of subsets of these sites can elicit a strong defect in translational control of the *osk* mRNA. This has been shown for translational activation mediated by the *osk* C region, where mutation of either the BREs or the type II binding sites causes a substantial loss of activation (Reveal et al. 2010). Thus, the full complement of binding sites is required for efficient translational activation. Second, in the reporter assays described here a high local density of Bru binding sites is required for detectable translational control. Two relatively distant copies (separated by a 88 nt spacer) of any of the candidate regulatory elements do not confer substantial repression, while four copies in a region of the same length do provide repression. Thus, evidence that Bru can bind an mRNA does not by itself demonstrate that Bru will have a major effect on its translation. We suggest that it is the combination of highly specific RNA binding by Bru and the presence of multiple Bru binding sites that together ensure that a target mRNA will be efficiently regulated.

MATERIALS AND METHODS

Protein purification

The RRM1+2 and RRM3+ Bru protein domains were expressed in *E. coli* using the pET3a vector for purification via the T7 tag. Pelleted cells from induced cultures were frozen at -80ºC, thawed and resuspended in 1x T7 tag bind/wash buffer from a T7 purification kit (Novagen), and lysed by sonication. Debris was removed by centifugation and the supernatent filtered with a 0.2 μm filter (Nalgene). Protein in the filtered supernatent was purified using the batch-wise method detailed in the manufacturer's protocol. A vivaspin spin column (Sartorius) was used to concentrate the protein in a final storage buffer of 50mM HEPES pH 7.9, 100mM KCl, 1mM EDTA, 10% glycerol.

Bru protein was expressed in *E. coli* using the pET15b vector, which provides a 6xHis tag for purification. Pelleted cells from induced cultures were frozen at -80 ºC, thawed and resuspended in histag buffer (20mM phosphate buffer pH 7.8, 500mM NaCl, 20mM imidazole, 10% glycerol), and lysed by sonication. Debris was removed by centifugation and the supernatent filtered with a 0.2 μm filter (Nalgene). Protein was loaded onto ProBond resin (Invitrogen) and eluted with an increasing concentration of imidazole to a final concentration of 300mM. Peak fractions were combined and concentrated by dialysis against PEG solution (25% PEG MW 15-20K, 200mM KCl, 1mM EDTA). Additional dialysis was used to equilibrate in protein storage buffer (50mM HEPES pH7.9, 100mM KCl, 1mM EDTA).

Selection

RNA for selection was prepared by transcription of a synthetic DNA template consisting of GATAATACGACTCACTATAGGGTTACCTAGGTGTAGATGCT $(N)_{50}$ AAGTGACGTCTGAACTGCTTCGAA where the random segment was prepared with equimolar amounts of the four nucleotides. Transcripts were produced using the Ampliscribe T7 polymerase kit (epicentre), and gel purified.

Prior to incubation with the selective protein, 415 pmol (2.5 x 10^{14} unique molecules) of the RNA aptamer pool was passed through a nitrocellulose filter (Millipore, HAQP01300) secured by a syringe filter apparatus (Whatman, 420100). The aptamer pool was incubated with the selective protein (full length Bru, RRM1+2, or RRM3+) for 30 minutes at room temperature in 50µl total volume of 1x SELEX binding buffer (20mM HEPES, pH 7.9, 100mM KCl, and 2mM $MgCl_2$). For the first four rounds, equimolar amounts of RNA and protein (200 pmol - 415 pmol) were used. For the final rounds of selection RNA was present at 5 fold molar excess. After each binding incubation, the reaction was again passed through a nitrocellulose filter and bound RNA was eluted by incubation for five minutes at 98°C in 200µl of elution buffer (7M Urea, 100mM NaOAc, 3mM EDTA). Eluted RNA was precipitated and resuspended in 20µL of water. 10µl of the resuspended aptamer RNA was used for a cDNA reaction with M-MLV reverse transcriptase (Invitrogen) and the products were amplified by PCR (forward primer: GAT AAT ACG ACT CAC TAT AGG GTT ACC TAG GTG TAG ATG CT, reverse primer: TTC GAA GCA GTT CAG ACG TCA CTT). The PCR products were then used for a further round of transcription, binding, cDNA synthesis and amplification.

The selection process was monitored using a filter binding assay (described below) (Fig. 2.8). Every three rounds of selection, the binding of the selective protein to

the current aptamer pool was compared to that of the initial aptamer pool as well as select previous aptamer pools. The selection was considered complete when binding reached a plateau. For RRM3+ and Bru eleven rounds of selection were required to reach completion. The RRM1+2 selection was complete earlier.

cDNAs from the selections were cloned using the TopoTA cloning kit (Invitrogen) and the inserts sequenced. The sequences were compared using the MEME program (http://meme.sdsc.edu/meme/meme.html or Bailey and Elkan, 1994). Tetranucleotide frequencies were determined using the search function of BBEdit 6.5 (Bare Bones Software), which identifies non-overlapping instances of the search string.

Transgenes

Transgenes with Bru binding motifs were all based on *UAS-GFP* (Reich et al. 2009). The *UAS-GFP-osk AB* transgene has been described (Reveal et al. 2010). For the initial set of reporter transgenes the binding motifs were placed at the ends of an 88 nt segment from the SV40 3' UTR, and cloned as BamHI-BglII fragments into the BamHI site of *UAS-GFP*, just after GFP. The final transgenes were further modified by replacing internal portions of the SV40 sequences with two additional binding motifs such that the four binding motifs were distributed at equal distances within the SV40 segment. The sequences from each of the fragments from the final clones, and from the SV40 control, are shown below. Bru binding motifs are underlined.

UAS-GFP-UGUUUUAUAUGU

TGTTTTATATGTGATGAGTTTGGGACAAACCACATGTTTTATATGTTGAAAAA
AATGCTTTATTTGTTGTTTTATATGTGCTATTGCTTCATTTGTAACCTGTTTTAT
ATGT

UAS-GFP-UGUUUGUAGU

TGTTTGTAGTGATGAGTTTGGGACAAACCACAACTGTTTGTAGTTGAAAAAA

ATGCTTTATTTGTGTGTTTGTAGTTGCTATTGCTTCATTTGTAACCTGTTTGTA

GT

UAS-GFP-UUGUCC

TTGTCCGATGAGTTTGGACAACCACAACTATTGTCCAGTGAAAAAAATGCTTT

ATTTGTGATTGTCCTGATGCTATTGCTTCATTTGTAACCCTTGTCC

UAS-GFP-UCAAUUGCAGU

TCAATTGCAGTGATGAGTTTGGGACAAACCACAATCAATTGCAGTTGAAAAA

AATGCTTTATTTGTTCAATTGCAGTTGCTATTGCTTCATTTGTAACCTCAATTG

CAGT

UAS-GFP-CAAUUUUAUAUGU

CAATTTTATATGTGATGAGTTTGGGACAAACCACCAATTTTATATGTTGAAAA

AAATGCTTTATTTGTCAATTTTATATGTCTATTGCTTCATTTGTAACCCAATTT

TATATGT

UAS-GFP-UAAAGUCUUCUA

TAAAGTCTTCTAGATGAGTTTGGGACAAACCACATAAAGTCTTCTATGAAAA

AAATGCTTTATTTGTTAAAGTCTTCTAGCTATTGCTTCATTTGTAACCTAAAGT

CTTCTA

UAS-GFP-SV40

CAGACATGATGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAA

ATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCCAGACATG

AT

Confocal analysis

GFP transgenic flies were grown at 25ºC. 2-3 day old flies were placed in well yeasted vials and incubated at 25ºC for another two days. Ovaries were dissected in PBS and fixed in a solution of 1200μL of PBS and 150μL 37% formaldehyde for twenty minutes with gentle mixing. The ovaries were then washed for one hour in four changes of PBT (1xPBS, 0.1% Tween 20). Quantitative data of immunofluorescence was collected using Leica confocal software from images collected by confocal microscopy using a single plane of focus. The GFP signal from nurse cell cytoplasm was sampled from three different locations in the egg chamber in each of 15 stage 9 or 10 egg chambers. Samples to be imaged for figures were stained with Topro (Molecular Probes) to label nuclei.

RT-PCR

Ovaries from 20 females, prepared as described above, were dissected in PBS and homogenized with a pestle. Total RNA from the ovaries was prepared using Tri Reagent-LS (Molecular Research Center, Inc.) according to manufacturer instructions. The isolated RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNAs were used in a quantitative real-time PCR with primers for either the GFP coding region (GFP-F,

TTTTCGTTGGGATCTTTCGAA; GFP-R, ACGGCGGCGTGCAAC) or *rp49* (rp49-F, GCGCACCAAGCACTTCATC; rp49-R, GACGCACTCTGTTGTCGATACC). The quantitative real-time PCR was carried out using the Power SYBR® Green Master Mix (Applied Biosystems) per manufacturer instructions in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The GFP cDNA sample levels were normalized using *rp49* cDNA sample levels. The real-time PCR was quantitated using the SDS software v2.2 (Applied Biosystems).

Flies used

Transgenic fly stocks were established by standard methods. Expression of UAS transgenes was driven by the *nosGAL4VP16* driver (Van Doren et al. 1998) or the *matα4-GAL-VP16* driver (Martin and St Johnston 2003), as indicated. $aret^{Z2286}$ was from M. Lilly (Sugimura and Lilly 2006) and $aret^{PA}$ from Trudi Schüpbach (Schüpbach and Wieschaus 1991).

RNA binding

UV crosslinking assays with ovarian extracts were performed as described (Kim-Ha et al. 1995) using RNAs uniformly radiolabeled with alpha ^{32}P-UTP. For the competition binding experiments the unlabeled competitor RNAs were present at 10-, 100- and 1000-fold excess.

RNAs for filter binding assays were synthesized with a T7 polymerase kit (epicentre, AS3107) and gel purified. 5' phosphates were removed with Shrimp Alkaline Phosphatase and the RNAs then labeled with gamma ^{32}P-UTP and T4 polynucleotide kinase.

Nitrocellulose (Whatman, 10-401-196) and nylon (Amersham Biosciences, RPN119B) filters were incubated in binding buffer for 30 minutes, placed together in a dot blot apparatus (Whatman, 10-447-900), modified as in (Wong and Lohman 1993), and pre-washed with 100µl of binding buffer. RNA (200 pM) was incubated with Bru (1.26-322 nM) for 30 minutes at room temperature in a 50µl volume of 1x SELEX binding buffer. The binding reactions were passed sequentially through the nitrocellulose and nylon filters, followed by a wash with 350µl of binding buffer. The filters were imaged with a BioRad phospho-imager (Molecular Imager PharosFX System). All assays were performed in triplicate. Radioactive signal intensities were measured and plotted for comparison using this equation: bound RNA/total RNA. The binding data for the *osk* AB RNA and the UGUUUUAUAUGU- and CAAUUUUAUAUGU-containing fragments were fit to a hyperbola: $y = (y_{max}[Bru])/(K_d + [Bru])$.

Acknowledgments

We thank Trudi Schüpbach and Mary Lilly for fly stocks, and members of the Macdonald lab and Brad Hall for useful comments on the manuscript. Brad Hall generously provided advice on the selections. This work was supported by grants from the NIH (GM54409, GM076536 and GM077040) and from the Welch Foundation (F-1654).

FIGURES

Figure 2.1. Bru protein structure.

Organization of Bru protein. The structure is shown schematically, to scale, with the three RRM RNA binding domains indicated. The subdomains of Bru used for selections, RRM1+2 and RRM3+, are shown.

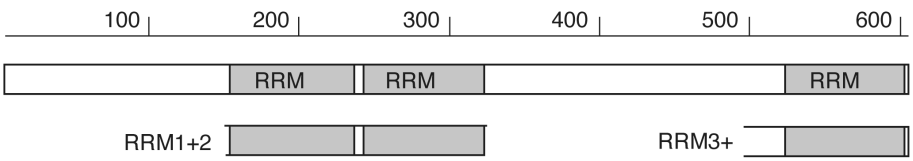


Figure 2.2. RRM3+ and Bru motifs.

Graphical representations of preferred binding motifs identified by in vitro selections. The height of each stack represents the information content at each nucleotide of the motif in bits. (A) The motifs identified from the RRM3+ selection. (B) The predominant motif identified from the Bru selection.

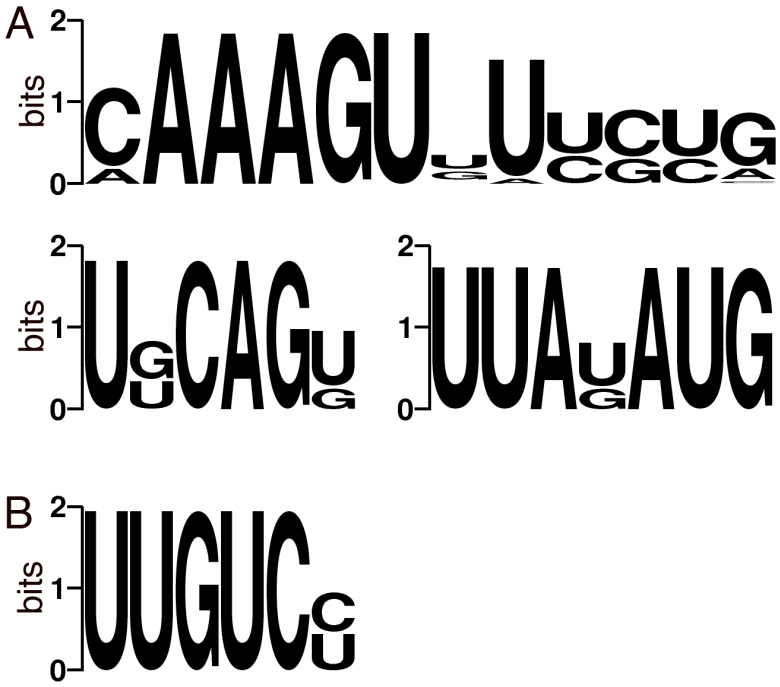


Figure 2.3. Motif distribution 3' UTRs of mRNAs.

The 3' UTRs of the indicated mRNAs are shown schematically, with motifs from the aptamer selections indicated. The full height bars are perfect matches to the motifs, while the half height bars have a single mismatch. The top five 3' UTRs are of Bru target mRNAs, while the bottom two 3' UTRs are from other mRNAs not known to be regulated by Bru.

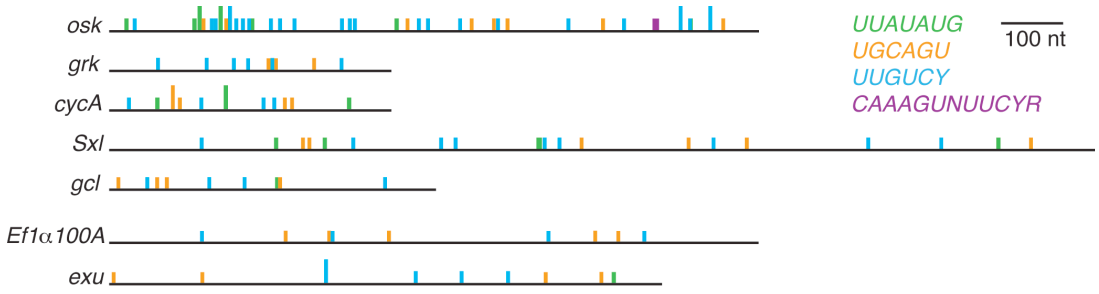


Figure 2.4. Translational repression by candidate regulatory sites.

(A) Schematic diagram of reporter mRNAs with the variable region indicated by a filled box. For the SV40 reporter, the variable region is only SV40 sequences. For the remaning reporter mRNAs the variable region has four copies of a candidate Bru regulatory site embedded in SV40 sequences.

(B-H) Examples of GFP levels in stage 10A egg chambers expressing GFP reporter transgenes with the *matα4-GAL-VP16* driver. All confocal images were taken on the same day at the same settings. The scale bar represents 75 μm. (B) Control GFP transgene with no anti-Bru aptamer binding motifs. The remaining image panels are for GFP transgenes with the Bru binding motifs indicated in the figure and described below. (C) UGUUUUAUAUGU is from the *osk* AB region, and consists of a BRE-like motif adjacent to a short U/G rich motif (like those from the RRM1+2 selection). (D) CAAUUUUAUAUGU is from the *cycA* 3'UTR, and consists of a BRE-like sequence adjacent to a short C/A rich motif (like those from the RRM1+2 selection). (E) UCAAUUGCAGU is from the *cycA* 3' UTR, and consists of a copy of the UGCAGU motif (from the RRM3+ selection) adjacent to a short C/A rich motif (like those from the RRM1+2 selection). (F) UGUUUGUAGU is from the *grk* 3'UTR, and consists of the UGCAGU motif (from the RRM3+ selection but with a single mismatch) adjacent to a short U/G rich motif (like those from the RRM1+2 selection). (G) UUGUCC is the type II Bru binding site, which appears three times in the AB and C regions of the *osk* 3'UTR. (H) UAAAGUCUUCUA is from the *osk* C region, and is a type III Bru binding site with a single mismatch relative to the longest aptamer motif from the RRM3+ selection. (I) Relative GFP levels in the nurse cell cytoplasm of stage 9/10 egg chambers for each of

the reporter transgenes. GFP levels (obtained from 45 measurements for each transgene) were normalized to the RNA levels (from 3 measurements for each transgene). Transgene RNA levels were normalized relative to *rp49* RNA levels. All of the reporter transgene mRNAs with candidate regulatory sites show reductions in GFP levels that are significantly lower than for the control ($p<0.0001$ by the Tukey-Kramer method).

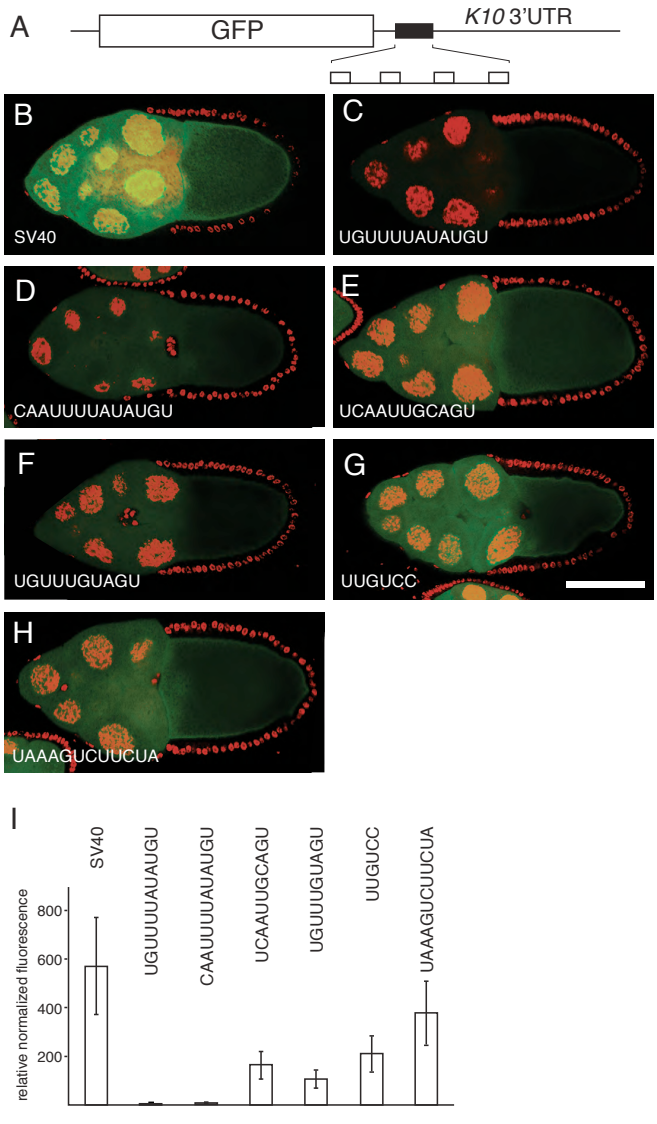


Figure 2.5. Translational repression of reporter mRNAs requires Bru.

A-G. The pairs of panels show GFP expressed from a reporter mRNA in *aret*$^{-}$/+ ovaries (A-G, left) or *aret*$^{-}$/*aret*$^{-}$ ovaries (A'-G', right). The identity of the Bru regulatory sites is shown below, with the relative increase in GFP level from mutation of *aret* indicated beneath the mutant panels. The scale bar represents 50 μm. In all cases the driver was *nosGAL4VP16*, which is active at early stages of oogenesis (the *aret* mutant ovaries arrest oogenesis and do not progress to the stage shown in Fig. 2.4). The transgenes are the same as those in Fig. 2.4, with four copies of a particular Bru binding motif as indicated.

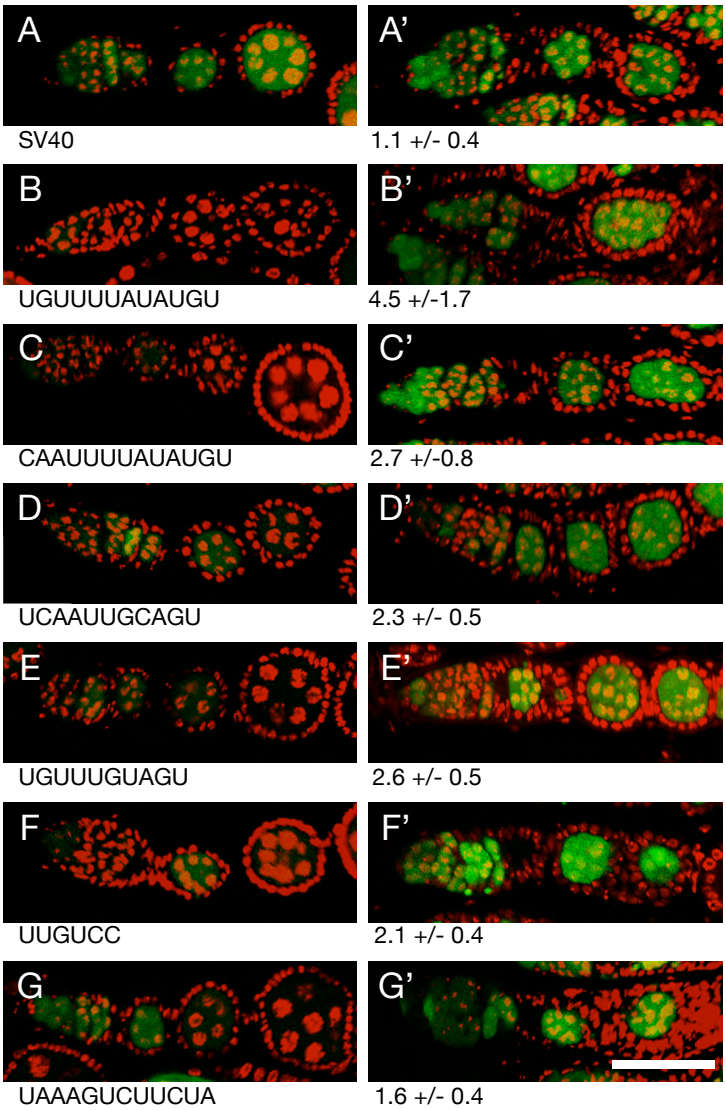


Figure 2.6. Bru binding to regulatory sites.

(A) UV crosslinking assay with ovarian protein and RNAs bearing four copies of the Bru binding sites embedded in SV40 sequences. The RNA probes are indicated at top by the identity of the binding site. The SV40 probe is SV40 RNA alone, and the *osk* AB probe is the AB region of the *osk* 3' UTR.

(B) Competition binding assay. Crosslinking assays of the type shown in panel A were repeated, with or without the presence of unlabeled competitor RNAs. The competitors are *osk* AB RNA, or the same RNA (all$^-$) with point mutations in BREs and type II Bru binding sites (Reveal et al. 2010). Exposures of the different rows are not equivalent, but were chosen to have similar binding signals in the absence of competitor.

(C) Filter binding assay with recombinant Bru and the RNAs used in panels A and B.

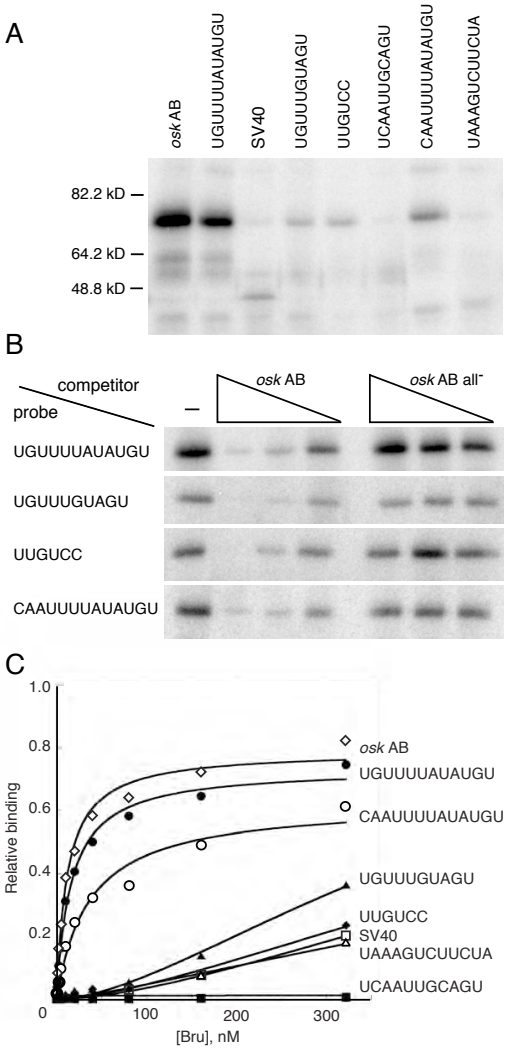


Figure 2.7. Models for Bru binding.

A. Combinatorial binding: different RNA binding domains of Bru interact with extended binding sites in the same substrate RNA. B. Independent binding: different RNA binding domains interact with different substrate RNAs. C. Bridging binding: different molecules of Bru bind to the same substrate RNA. For simplicity, the third Bru RNA binding domain identified from the Bru selection is not shown as its position in the protein is unknown.

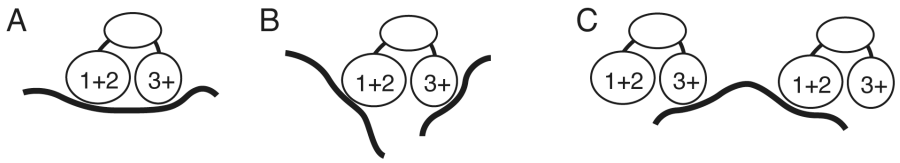
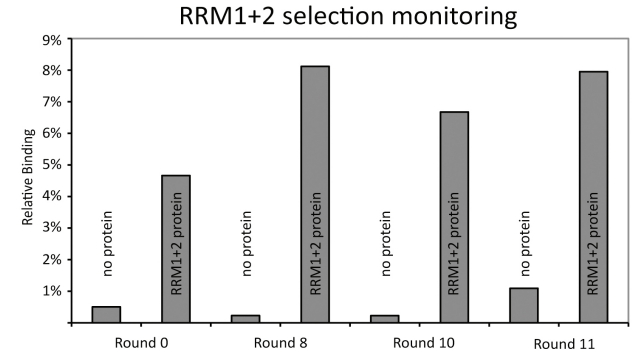
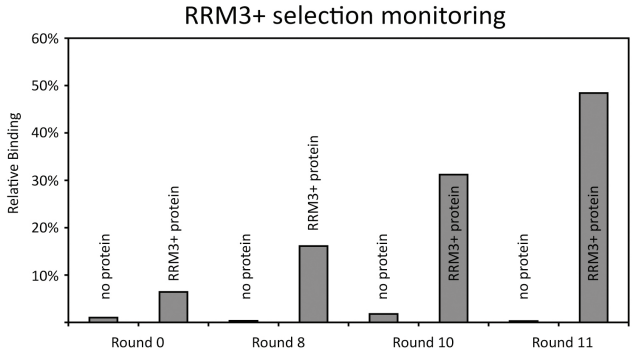
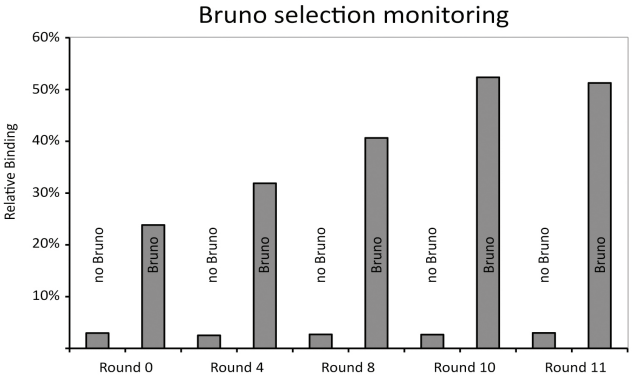


Figure 2.8. Monitoring of selections

Selections were monitored using a double filter-binding assay. An equamolar amount of selective protein and radiolabeled RNA pools were incubated for one hour and then passed sequentially through a nitrocellulose filter, to capture protein bound RNA, and a nylon filter, to capture unbound RNA. The amount of bound RNA was compared to total RNA to get a relative binding value. RNA not incubated with protein was also passed through both filters for comparison. The RNAs used were the initial round RNA, the final round RNA aptamers, the round prior to the final round, and three rounds prior to the final round.







TABLES

Table 2.1. Highly enriched tetranucleotides in the RRM1+2 and Bru selections

tetranucleotide	n	copies in *osk* AB (127 nt)	copies in *osk* C (76 nt)	copies in *osk* 3' UTR (1041 nt)	copies in Bru aptamers	rank among Bru aptamer tetranucleotides
U/purine rich (only U or purine)						
UUAU	37	5	3	11	74	12
UAUG	24	5	2	9	23	55
UGGA	19	-	-	3	1	218
UGUU	17	7	1	14	103	5
UUUA	16	4	2	16	57	18
C/A rich (at least 3 of the 4 nt are A or C)						
AUCA	27	-	1	8	11	90
CAAA	23	-	-	15	20	147
UCAA	20	-	-	9	15	74
UCAC	20	-	-	2	11	90
AAAA	18				3	181
AAAG	17	-	-	5	3	181
CAUA	16	-	-	2	17	71
Other (don't fit in either group above)						
UAUC	22	-	-	3	35	32
UUCU	19	2	-	11	66	15
AGCU	18	-	1	5	3	181
UUCA	17	-	-	2	10	95
UCUG	16	1	-	7	40	25
UCUA	16	1	2	5	38	28
UCUU	15	1	1	6	92	7
GUCU	15	1	1	11	81	9

Tetranucleotides appearing at least 15 times in the RRM 1+2 aptamers are shown.

Table 2.2. Nucleotide composition of aptamers and *osk* 3'UTR regions

	nucleotide composition			
RNAs	A	C	G	U
osk 3' UTR	29%	18%	18%	36%
osk AB region	18%	10%	17%	54%
osk C region	28%	9%	16%	47%
RRM 1+2 aptamers	28%	21%	19%	32%
RRM 3+ aptamers	27%	21%	19%	33%
Bru aptamers	14%	23%	13%	50%

Table 2.3. RRM1+2 aptamers

aptamer	Aptamer sequence
8.2	UUCUACAGGAUGUGUGCAGUCGGAUUCCCAUAAUCCUUUCAUACCUAGCA
8.3	UUUAAUCACUUACGAGAGAAUCAAUCUCUCAUACGAAAUUAUGCAAUGUA
8.4	GCUUAUAGGUCAAAGCAACCAUUUAGUUCUACCGGACGGUUGGAAAUUAU
8.5	UAUGUUAUGCAAGAGUUCAAAAAACUCAGCUUCUGUCUGCCUGGAGCUCUA
8.6	GGAUCAUUCACAUAAAAGGCUGAUCAAAGUGUUGUAUCUUAUCACCUCAG
8.7	GGAUCAUUCACAUAAAAGGCUGAUCAAAGUGUUGUAUCUUAUCACCUCAG
8.9	GGAUCAUUCACAUAAAAGGCUGAUCAAAGUGUUGUAUCUUAUCACCUCGG
8.10	UAUGUUAUGCAAGAGUUCAAAAAACUCAGCUUCUGUCUGCCUGGAGCUCUA
8.11	CUACGUAAAAUUUCUUGUGUAGUCACUUCUAAAGUCAGUAAGUGCAAAUC
8.12	AAUUGCCAAUCCGUCACGACCGUUAGACCGGUAGUCUACAAUGUUUGUAU
8.13	AUGACGUACGCAUUAUAUCUUUGUCGACGGUGUAUCACUUCUCUUUGCCC
8.14	UAUGUUAUGCAAGAGUUCAAAAAACUCAGCUUCUGUCUGCCUGGAGCUCUA
8.15	AUUUAGUAGUGAGGUCUCCAUAGCCACGUUAUCCAGUACUAUAUUUUAGU
8.17	GUCUUGUUUUAUGUUUUACACGAGUUUUAGAAACUCAAACUGGAAAGCGA
8.18	GCAAAAGCCGGUCGCGCUAAGUAUAGCAUCAUUUCUCUAAGCAUAUGUAA
8.19	UAUGUUAUGCAAGAGUUCAAAAAACUCAGCUUCUGUCUGCCUGGAGCUCUA
8.20	UAUGUUAUGCAAGAGUUCAAAAAACUCAGCUUCUGUCUGCCUGGAGCUCUA
8.21	CUACGUAAAAUUUCUUGUGUAGUCACUUCUAAAGUCAGUAAGUGCAAAUC
8.23	UAUGUUAUGCAAGAGUUCAAGAAACUCAGCUUCUGUCUGCCUGGAGCUCUA
8.26	AUUUAGUAGUGAGGUCUCCAUAGCCACGUUAUCCAGUACUAUAUUUUAGU
8.27	GGAUCAUUCACAUAAAAGGCUGAUCAAAGUGUUGUAUCUUAUCACCUCAG
8.28	GGAUCAUUCACAUAAAAGGCUGAUCAAAGUGUUGUAUCUUAUCACCUCGG
8.29	AUUUAGUAGUGAGGUCUCCAUAGCCACGUUAUCCAGUACUAUAUUUUAGU
8.30	AAUUAGCUCGAUACAUUAUAAUUUACCAAAUCAUGCAGGAGUCGAUUUGU
8.31	AUGACGCACGCAUUAUAUCUUUGUCGACGGUGUAUCACUUCUCUUUGCCC
8.32	GGAUCAUUCACAUAAAAGGCUGAUCAAAGUGUUGCAUCUUAUCACCUCAA
8.33	UAUGUUAUGCAAGAGUUCAAAAAACUCAGCUUCUGUCUGCCUGGAGCUCUA
8.34	UUUAAUCACUUACGAGAGAAUCAAUCUCUCAUACGAAAUUAUGCAAUGUA
8.37	GCUUAUAGGUCAAAGCAACCGUUUAGUUCUACCGGACGGUUGGAAAUUAU
8.39	UAUGUUAUGCAAGAGUUCAAAAAACUCAGCUUCUGUCCGCCUGGAGCUCUA
8.45	AUUUAGUAGUGAGGUCUCCAUAGCCACGUUAUCCAGUACUAUAUUUUAGU
8.46	AGGUCUAAGCAGGAACGCCAAAACCUGGAAAUUAGCAUCCCUUCUCAUA
8.50	AGGAGGUAUUACAAACCGUUUGGACUAGCUUCUUUUCAAAAAAUUCACCC

Table 2.4. RRM 3+ aptamers

Aptamer	Aptamer sequence
4.1	AUAUGAUUGCUGCAGUUCUUGAUUCCCUCAAAGUAAUCUGUCCACCUGGC
4.3	GCUCAAAGUUUCCUGUAGCAGGUUUCCCAAAAAUGAAUAGUUCCUUCUUA
4.4	GGGUUACUUUUGUGCUUGUAGACCAAAGUGUUGCCUCUACCCUCAACAUA
4.5	AUUAGUUCAGUACUCCCAAUGCUAAAUCGAUUAUCCGAUACACCUUAGGA
4.8	GCUCAAAGUUUCCUGUAGCAGGUUUCCCAAAAAUGAAUAGUUCCUUCUUA
4.11	AAAACCUUCAUCCAAAUGCGUUACUAUGGUUUGAGAAAAGUCUUCCGCUC
4.12	CCACUUAUUGAAAUAUAGCCUUGCAAACAAGGCCGUAUUGUGAUUGACAU
4.13	UAUCUGGUUGAAAAGUUUUCCGCAAAAGGCAUCGAAGGAUAUUAUCACAA
4.16	GCUCAAAGUUUCCUGUAGCAGGUUUCCCAAAAAUGAAUAGUUCCUUCUUA
4.18	AAUGGGCCAUAUUUAGAUGAGCCAAAGUGUUGUAGAAUCUACUAACUUUU
4.21	CUGAUUCAGUAUGGGAUGGCUAUUACCGCCACUCCGAAGUGUUUAUUGAU
4.22	UUCUAUCUGACUCGGAUUUAUAUGAUCAUAUAUCCUCAGGUGUUGUCAGA
4.25	CUGAUUCAGUAUGGGAUGGCUAUUACCGCCACUCCGAAGUGUUUAUUGAU
4.26	UAGCUUCUUGCUAAAUGUUAUAUGACUUCCUCUGUCUGAUGCAGGAGCUU
4.27	UAAUCGAGGACAUAGAGUUUGCAGUCCUCACCGGUACAGCCAGUCAUC
4.28	UAGCUUCUUGCUAAAUGUUAUAUGACUUCCUCUGUCUGAUGCAGGAGCUU
4.29	GGGCCUACAAGCCAAAGUGUUGCGUCCACACAUUGGUUUAAUCACUCAAG
4.30	GCUCAAAGUUUCCUGUAGCAGGUUUCCUAAAAAUGAAUAGUUCCUUCUUA
4.33	AAUGGGCCAUAUUUAGAUGAGCCAAAGUGUUGUAGAAUCUACUAACUUUU
4.34	CAGAUUCAGUAUGGGAUGGCUAUUACCGCCACUCCGAAGUGUUUAUUGAU
4.36	GGGCCUACAAGCCAAAGUGUUGCGUCCACACAUUGGUUUAAUCACUCAAG
4.37	UUUUAAAGAUCUCUGCAGUGUGCGAGCAAAGCUAAACAAGCUUUAUACAA
4.38	GCUCAAAGUUUCCUGUAGCAGGUUUCCCAAAAAUGAAUAGUUCCUUCUUA
4.39	UAGCUUCUUGCUAAAUGUUAUAUGACUUCCUCUGUCUGAUGCAGGAGCUU
4.40	UAGCUUCUUGCUAAAUGUUAUAUGACUUCCUCUGUCUGAUGCAGGAGCUU
4.42	UAUCUGGUUGAAAAGUUUUCCGCAAAAGGCAUCGAAGGAUAUUAUCACAA
4.45	CCGUAAUUAUCUAUUCUACAGCUUAGACUCACGAAUUAUAUGUAUCAGCA
4.46	CUGAUUCAGUAUGGGAUGGCUAUUACCGCCACUCCGAAGUGUUUAUUGAU
4.48	GUCCAUAGCAAAUGCAGUUCGAUUCCCACACUAUGGCUAUACACUUAGAA
4.49	GCUCAAAGUUUCCUGUAGCAGGUUUCCCAAAAAUGAAUAGUUCCUUCUUA
4.50	CUGAUUCAGUAUGGGAUGGCUAUUACCGCCACUCCGAAGUGUUUAUUGAU
4.51	GCUCAAAGUUUCCUGCAGCAGGUUUCCCAAAAAUGAAUAGUUCCUUCUUA
4.54	AAUGGGCCAUAUUUAGAUGAGCCAAAGUGUUGUAGAAUCUACUAACUUUU

Table 2.5. Bru aptamers

Aptamer	Aptamer sequence
Bru.1	AUCAUAGUUUAUACUCCUCUUUGUCUUCUGUGUACCGUAUUGUAAAUUUA
Bru.2	UUGUGGGGGUCAAUUCCUGUCUCAGGCCCCGUGUCUCACACCUUGUACAC
Bru.4	CUCUUACUGCCACCUCCUGUUGUUUAUUUGUUUUUUUUUUCUAAAAUUAGUUC
Bru.5	AUCUGCACUACUGUCUUGUCUUGUAUCUGUCUUCGUAUGUUUUUUCCUA
Bru.6	GUGUCCUGUCCCUCUCUCUUUAUUGUCUAUGCUACGAUCCUUCCCA
Bru.7	UGCCCUACCCUCUUUUUAUUUGUCUUUGUCCUCCAUGUAUUAAUAUUUCU
Bru.8	UCUAUUCUUCUUUAUUUUUUUGUUAUUUGUAUUGUCUUUGUGUGAAUCAGU
Bru.10	AUCUUUCUCGUUUCUAUUCGCUUUUUUUUUUUUGUGUUUUGUCCUCUGUUUGUC
Bru.11	AAUCAAUUACUUGACCCAAUUCGUUUUUUGUGUUUGUCCAAUCGAUAUGC
Bru.12	ACCUAUCCUUGUCUUGUUUUUCUUUUUUUGUCCUCUCUAUCUCGUGUCCUGCUU
Bru.13	AAUGACCUCCGUCCUGUCUUAUUUUAAAUUUGUGUUGUAUAUUCUUGUCC
Bru.14	UUCUAUACUCUAUUAUUUGUUUUUGUCUAUCCUCUCUGUCUAUAAUGUUCC
Bru.15	CCAACUACGCAUCCGCUAUUUUUGUUGUUUUUGUUUUUGAUUUCGUAAGC
Bru.16	UCUGCUCGCUACCUGUUUUUGUUUUUGUUUUUGUUUCUUCUUUAUCCACGUGUUUGUCCUCUA
Bru.17	UAUUGUAACCUUCUUUCUGUAUAUGUCCCAUUGUUUUUUUUUCUUCCACCCCUAUCCGUACUGAGCUCU
Bru.18	AUACCCUCUUCCGUCUGUAUUCCUCGUUUUGUCCUCUUUCCUUCUGUGC
Bru.19	GUCCCAUUUUGUUAUUGUCCAUUAUUUUUUUUUUUUUUUUUUUUGUUACUAUUUGUUGCU
Bru.20	UACUUUGUGAGGGUUAUUUGUCCGGACCCUGUAAGUGCCUAUACAGUGUA
Bru.21	CUUGUCCCUUUGUUUUAACAAUUGUUUUGUCUUUUCGAGCCUUAGUUA
Bru.22	AAUCAUUCUACAAUGCUCCUUACUUUUAAAUUUGUGUAUCUCU
Bru.23	CGUCGUCAAUUGCGUCUAAUUUUGUUUUGUUUCUGUCCUUUUCGUACG
Bru.24	UAUCAACUUGUCCAUUCCAUCCGCCUGCCUUUUUGUGUUGUACUCUCGUA
Bru.25	UUUUUGUAUUGUUUCCUCCAAUUUUUGUCUAUGUUUUCGUCUUUGUCUCUG
Bru.26	UCUCCCCCCUCUUGUCCUUUGUUUUUUUUAUAUUUUCGUUUUUCCUCCGCC
Bru.28	AUCGUUUCCAUUUUCUGUUUAUUCUCUGUGUCCAUAUUCAUUUCCUUCAU
Bru.29	UUGUGGGGGUCAAUUCCUGUCUCAGGCCCCGUGUCUCACACCUUGUACAC
Bru.30	GCAUCCCAUGUUAUAUGUCCUCCUCCUUCUCUGUUUUUCUCUCUCCCUAACAGC
Bru.31	GUCUUCACCUCCAAUUAUAUUUUGUCCAAUUUUGUAUUUUGUCUAUCUGU
Bru.32	AUGAAUGUUAAUUAACUUGUGUACCUCUCUAUCAUUUGACACUUUGUUU
Bru.33	UAAUAAGGUUUAGCGCUUGAUGUCCUGAGCGCGGCACCUAAUAGACCUAC
Bru.35	GUAACCUAUAUUUAUUGUUUUUGUCUUUGUCCCUAAUCUAUUACUCAACU
Bru.36	AUGCCAACGUCUGUGUCUAUUUGUUUAUGUAUAUUUGUCUUUAUUUGUU
Bru.37	CUCAUCCAUACUGUCCUAAUAUUUAUUGUCCAUUGUCCUCCAAACCUUUUUUUUUUUUCUCUUUAU
Bru.39	CUUUAUGCCACUCGUUUGUCUCUGUCCUCUUAAAUUUUCUAAGCCUUUUC
Bru.40	UAAUACUUCUCCAUGCCUUCAUAAUUUUGUCCAUAGUCCGUUUUUUUGUUUGUUAUUGUCCCAGUCU
Bru.42	AUUUCACUGUCCUACCUAUAUUUUUUUUUUUUUUUGUUAUUUGUCCUAUAAGCACAUC
Bru.43	GCACUCUUAACCUUUUGUUUUUGUCCCCUCUCGUCCAACGUUCGAACACC
Bru.44	UCCAUAUUUUUUGUUGUGUCCUUUUUUUGUUUUUGUUGUCCAUUAUUAUUGU
Bru.45	CCAUUUUAUUUGUUUUUGUCCGUUUGUGUUUGUGUUCCUUCCGCAGUAUCU
Bru.46	GCCUGGUCUCCUUGUCUCGUUUUAUUGUCCUUUUUCUCCAUUCGCAUCUC
Bru.47	UACCAUUUACCAACCUCUAAAUUGUCUGUCUCGUUUAAUUUUUUUUUUUUUUUCAGU
Bru.48	UUCCCAUCUCCCGAUCUUAUUGUCUUUCCGUCUAUUGUUAUCAGUUCUUG
Bru.49	AUUCUCCAGACUACCUAUCUUUUUUUUUUUUUUUUGUUUAUAUGUGUGUUGUUAAUCU
Bru.50	UCAACCAUUCCUGUGUAUUUGUAUUUGUUUUUUAAUGUUAUGAUCGUGC
Bru.53	CAAAUUCGUUUUUGUUUCCUUUUUUUGUUUUUUUAAUGUGUAUUUUCUGUA
Bru.54	UGUCCUACCACUCUGCCCUUCCUUUUUGUCUUUAUCGUAUUUUAUCU
Bru.55	UUGUCAACUCUCCUCUUGUGUUUAUUGUCCUCGUCCAAAAGCUUCCGUUC
Bru.56	ACAAUUUCACGCCUCUUUUUUUGUCUUUUUCUGUAAUUUUGUUU
Bru.57	CAUUAUUGUCCUGUCCCAUGUCUUGUUAUAUUAUUGUU
Bru.61	GCUACUGCCUUUCUAUUAUUGUCUCUCUGUGUUAAAUCUUUGUCCUCUGC
Bru.62	CACUAUCACGACGGUUAACGUCUGUUGUCCAAUCUAUUAUUAACCGACCUU
Bru.63	CACUCUUCUGUUUUUGUAAUUGUCUCUCUCUCUUGUCCUCUUUUUCUCCAGUCUUAAAUUUGUUGUCCCCUGUCCCCUUA CUCUUACUC
Bru.65	UUUAUCUAUCCUUAUUUUUGUCUUGUCCUCAUUUUUUCUCCAGCCUCCGC
Bru.66	ACUUGAUUACUGGGAGGUUUUAUUUGUUGUAAUUGUCCAUUUUGUCUCCG
Bru.67	CAUAUGCUUUGUCCCGUCUUCUGUGUUUGUCCCUCAAUUUCUCCCGUUCU
Bru.68	CAUAAUUUCUCUGUUUUUGUCCUCUAAGUUUAUUUUUGUUUUUCGAUGUA
Bru.70	ACUGAAUCUCCGCCUUUCUGUGUUUGUCUUUUGUGUUUUGUUAUUAGUAUA
Bru.71	CCUUCCUAUAUCUCAAUUCCCUUUUGUCUGUCUUCUUUUUAUGUAUCCGC
Bru.72	UUCUUCUACCGCCAUCCCACCUUAGCGCAUUGUGAGCGCUCUCUAUAUU

Table 2.5 continued

Bru.75	CUAAUAUCCGACCCAUUGUAUUACUUGUCCCUUCUCUUUUGUCUUAUUCC
Bru.76	UGUAAGCUCACCUUUGUCUUUGUCUUAUAUGUUUUGUUUCGUAAGUAUGG
Bru.77	CCAAUCCUACUGUGUUUUUCUUUAUUCUGUCUUGUCCAUAUAAUAGACGU
Bru.78	AAGUAUGGUUAAUAAUUUGUGUACCUCUGCUUUAUUUACACAUGCAUUUA
Bru.80	CUACUUAUUCUCGUCCCUCGUCUUCUUUUUUAAUCGUUUUCCUCAAGU
Bru.81	ACUAUCGUCUCGGCAACCGUCUAUUGUCUUUUUUUUUUUUUGUUAGUCUUUCCCCUUCUUUUAUUUUUGUC
Bru.82	UAUUAACCUUUUCCGUCCGUUUUUUUUUUUGUCUUUUGUAAGUUCUCUGUGGA
Bru.84	UUCGACAUCUCCUUUAUUUGUCCGUCCUCAUGUUUUUAUUUUUGUACUCC
Bru.85	UAGUUGACCUAAUUGUUUUUAUACAUCUACAUUUGUCUUUGUUUCCUCUCU
Bru.86	UCGUGGGUUUAUGUUGUGUUUUAAGCCGUGUCAAUGCCUUGGUCUGCUUU
Bru.87	UCUAUCUGCCCAUUGUCUUUGUCCGCCUACGUUUCUGUUUUCCGGCAUUC
Bru.88	UCCUAGCCCAAUUAUUCCUAUUCUUCUUGUGUUGUCUAUUAGUUUGAAC
Bru.89	UGUUAAAUCCUAUACCCUCCUCGUCUUGUCUCAUCAUAAAAAUCCUGCUU
Bru.90	UCCGUUUUGUUUUUUAUUUUUGUCUCAUGUCCCAAGUUCACGUUUUUCC
Bru.91	CCGUUUGUCUUGUUUGUCCUCUUUGUUUGUUUUUUCUCUUCCCUAUCCCU
Bru.92	UAACACCUUAUUGUCUUGUCUUUAAUAUAUUGUCUUUUUGUUCUCGGCAU
Bru.93	CAGUAAAGUGCAAACGUUUUUUCGUCCCUAUUUUAUUUGCACACUGACC
Bru.94	CUCUCCUGUCUUCUCCUUUUAUGUGUUUUUAUGUUUUUUUUUUUUUUCCCUAGUUC
Bru.95	UCCGUGUGUUAUUCUUCUUGUCUUCUGUCCGUUUUUAUGCUCUCUAAAUA
Bru.96	CGCUUUAUAUUGUUUCCUUUAUUGUUUUUUGUCUGUCUUUUUUUUGUGC
Bru.97	UUUGCGCAGAAAGUCCCCAACUCGUCCCGUCCAUAUCUUCUGUUCCUGCU
Bru.98	UAAUCUUCUACCAUUGUCUUGUCUUUUUUUUUUUCCUGUCCAAAUCGAUCAAC
Bru.99	UGUCUAUAUCUUUAUUGUCUCUUCCUUUUUUCUGUCCUCAUAUUAUUGUUC
Bru.100	UACUAUUACAUUUAUACCGAUUACCAAUUUUUGUCCUUGUUUUGUCCCUU
Bru.101	CAACGUUUUUUUUUGUCUCAGUGUUUUAUUGGGCUAUCUACGCUACCUUAA
Bru.102	AUCCGUAUUAAUUGUUUUUGUCCCAUCUUUUGUGUU
Bru.103	CACCGAUUUUCUUGUUAUCUUCCAUAUAUAUGUCCUCUAUUUUGUCCUUCCC
Bru.104	UAGCCCAGAGUGUCGCCCUGUCCCAACCAAUUUGUACUUGUCCUCGCCCUUUAAUAUAUUAGCCAGA
Bru.106	UCCACCCAUAUCUUUGUUUGUGUCUUUAUGUGCUUCGUUUUUUUCUAUUU
Bru.107	AUAAUUCCCAUUCUGUUUUCAUAUUUUGUGUUGUCCCUAUCAUAUUCUUA

Table 2.6. Frequency tetranucleotides in RRM1+2 and Bru selections

tetranucleotide	RRM1+2 count	RRM1+2 rank	Bru count	Bru rank
aaaa	18	10t	3	181t
aaac	12	32t	2	192t
aaag	17	12t	3	181t
aaat	9	60t	12	84t
aaca	0	220t	4	180
aacc	4	136t	10	95t
aacg	1	192t	5	147t
aact	10	49t	6	133t
aaga	8	69t	0	241t
aagc	6	101t	6	133t
aagg	6	101t	1	218t
aagt	9	60t	9	105t
aata	0	220t	11	90t
aatc	10	49t	15	74t
aatg	3	155t	7	121t
aatt	10	49t	35	32t
acaa	2	172t	3	181t
acac	1	192t	9	105t
acag	1	192t	2	192t
acat	7	90t	6	133t
acca	3	155t	7	121t
accc	1	192t	8	120
accg	5	122t	6	133t
acct	8	69t	25	51
acga	6	101t	2	192t
acgc	4	136t	3	181t
acgg	5	122t	1	218t
acgt	8	69t	9	105t
acta	5	122t	7	121t
actc	9	60t	13	77t
actg	1	192t	10	95t
actt	6	101t	12	84t
agaa	3	155t	1	218t
agac	1	192t	3	181t
agag	10	49t	0	241t
agat	0	220t	0	241t
agca	7	90t	1	218t
agcc	5	122t	7	121t
agcg	1	192t	4	164t
agct	18	10t	3	181t
agga	4	136t	0	241t
aggc	6	101t	2	192t
aggg	0	220t	1	218t
aggt	8	69t	2	192t
agta	10	49t	5	147t
agtc	5	122t	5	147t
agtg	11	40t	5	147t

Table 2.6 continued

agtt	11	40t	10	95t
ataa	8	69t	9	105t
atac	4	136t	10	95t
atag	7	90t	4	164t
atat	8	69t	32	37t
atca	27	2	11	90t
atcc	7	90t	18	66t
atcg	0	220t	7	121t
atct	11	40t	29	42t
atga	3	155t	3	181t
atgc	11	40t	10	95t
atgg	0	220t	2	192t
atgt	14	21t	24	52t
atta	11	40t	24	52t
attc	8	69t	26	49t
attg	1	192t	39	26t
attt	14	21t	71	14
caaa	23	4	5	147t
caac	2	172t	13	77t
caag	8	69t	2	192t
caat	6	101t	20	60t
caca	6	101t	5	147t
cacc	7	90t	12	84t
cacg	6	101t	4	164t
cact	6	101t	9	105t
caga	0	220t	4	164t
cagc	8	69t	2	192t
cagg	3	155t	2	192t
cagt	6	101t	9	105t
cata	16	15t	17	69t
catc	2	172t	12	84t
catg	1	192t	7	121t
catt	13	29t	26	49t
ccaa	3	155t	20	60t
ccac	4	136t	7	121t
ccag	4	136t	6	133t
ccat	7	90t	33	36
ccca	1	192t	19	63t
cccc	0	220t	9	105t
cccg	0	220t	6	133t
ccct	1	192t	28	45
ccga	0	220t	5	147t
ccgc	0	220t	9	105t
ccgg	4	136t	2	192t
ccgt	3	155t	23	55t
ccta	1	192t	27	46t
cctc	6	101t	43	23t
cctg	9	60t	20	60t
cctt	2	172t	48	21

Table 2.6 continued

cgaa	2	172t	1	218t
cgac	4	136t	4	164t
cgag	3	155t	1	218t
cgat	2	172t	7	121t
cgca	3	155t	5	147t
cgcc	1	192t	8	117t
cgcg	1	192t	1	218t
cgct	1	192t	7	121t
cgga	3	155t	1	218t
cggc	0	220t	4	164t
cggg	0	220t	0	241t
cggt	7	90t	1	218t
cgta	4	136t	9	105t
cgtc	1	192t	22	58
cgtg	0	220t	8	117t
cgtt	6	101t	24	52t
ctaa	4	136t	12	84t
ctac	5	122t	19	63t
ctag	2	172t	2	192t
ctat	4	136t	43	23t
ctca	12	32t	16	72t
ctcc	4	136t	30	40t
ctcg	7	90t	17	69t
ctct	14	21t	52	19
ctga	6	101t	4	164t
ctgc	8	69t	13	77t
ctgg	10	49t	2	192t
ctgt	8	69t	50	20
ctta	10	49t	18	66t
cttc	14	21t	38	28t
cttg	2	172t	34	35
cttt	8	69t	75	11
gaaa	7	90t	1	230
gaac	1	192t	2	192t
gaag	0	220t	0	241t
gaat	2	172t	3	181t
gaca	0	220t	2	192t
gacc	2	172t	8	117t
gacg	8	69t	2	192t
gact	1	192t	1	218t
gaga	2	172t	0	241t
gagc	8	69t	4	164t
gagg	5	122t	2	192t
gagt	10	49t	1	218t
gata	1	192t	1	218t
gatc	12	32t	4	164t
gatg	1	192t	2	192t
gatt	2	172t	5	147t
gcaa	14	21t	2	192t

Table 2.6 continued

gcac	0	220t	5	147t
gcag	3	155t	2	192t
gcat	6	101t	7	121t
gcca	6	101t	5	147t
gccc	3	155t	9	105t
gccg	1	192t	1	218t
gcct	8	69t	13	77t
gcga	1	192t	0	241t
gcgc	1	192t	5	147t
gcgg	0	220t	1	218t
gcgt	0	220t	1	218t
gcta	1	192t	6	133t
gctc	9	60t	6	133t
gctg	6	101t	0	241t
gctt	11	40t	10	95t
ggaa	5	122t	0	241t
ggac	3	155t	1	218t
ggag	10	49t	1	218t
ggat	8	69t	1	218t
ggca	0	220t	4	164t
ggcc	0	220t	2	192t
ggcg	0	220t	0	241t
ggct	6	101t	1	220
ggga	0	220t	2	192t
gggc	0	220t	1	218t
gggg	0	220t	2	192t
gggt	0	220t	4	164t
ggta	2	172t	0	241t
ggtc	8	69t	4	164t
ggtg	3	155t	0	241t
ggtt	2	172t	6	133t
gtaa	3	155t	12	84t
gtac	7	90t	10	95t
gtag	6	101t	0	241t
gtat	12	32t	23	55t
gtca	5	122t	5	147t
gtcc	0	220t	79	10
gtcg	6	101t	2	192t
gtct	15	19t	81	9
gtga	4	136t	3	181t
gtgc	2	172t	6	133t
gtgg	0	220t	4	164t
gtgt	11	40t	39	26t
gtta	13	29t	21	59
gttc	10	49t	13	77t
gttg	8	69t	15	74t
gttt	5	122t	94	6
taaa	8	69t	11	90t
taac	0	220t	10	95t

Table 2.6 continued

taag	4	136t	13	77t
taat	6	101t	29	42t
taca	5	122t	9	105t
tacc	4	136t	19	63t
tacg	8	69t	5	147t
tact	4	136t	27	46t
taga	2	172t	2	192t
tagc	9	60t	5	147t
tagg	4	136t	0	241t
tagt	12	32t	9	105t
tata	11	40t	30	40t
tatc	22	5	35	32t
tatg	24	3	23	55t
tatt	9	60t	91	8
tcaa	20	6t	15	74t
tcac	20	6t	11	90t
tcag	9	60t	7	121t
tcat	12	32t	17	69t
tcca	8	69t	36	31
tccc	2	172t	37	30
tccg	1	192t	27	46t
tcct	1	192t	74	12t
tcga	5	122t	6	133t
tcgc	1	192t	6	133t
tcgg	7	90t	2	192t
tcgt	0	220t	32	37t
tcta	16	15t	38	28t
tctc	11	40t	60	17
tctg	16	15t	40	25
tctt	15	19t	92	7
tgaa	0	220t	4	164t
tgac	3	155t	5	147t
tgag	4	136t	4	164t
tgat	6	101t	4	164t
tgca	14	21t	6	133t
tgcc	12	32t	11	90t
tgcg	0	220t	2	192t
tgct	2	172t	13	77t
tgga	19	8t	1	218t
tggc	0	220t	0	241t
tggg	0	220t	6	133t
tggt	0	220t	3	181t
tgta	14	21t	35	32t
tgtc	12	32t	137	3
tgtg	2	172t	32	37t
tgtt	17	12t	103	5
ttaa	2	172t	29	42t
ttac	5	122t	16	72t
ttag	14	21t	10	95t

Table 2.6 continued

ttat	37	1	74	12t
ttca	17	12t	10	95t
ttcc	1	192t	47	22
ttcg	0	220t	18	66t
ttct	19	8t	66	15
ttga	0	220t	7	121t
ttgc	4	136t	5	147t
ttgg	3	155t	3	181t
ttgt	13	29t	196	1
ttta	16	15t	57	18
tttc	5	122t	64	16
tttg	9	60t	124	4
tttt	10	49t	152	2

Table 2.7. Structural features of RNAs used for binding assays

				# of base pairs in binding site		
Binding site[a]	ΔG[b]	copy number[c]	size	G-C	A-U	G-U
UGUUUUAUAUGU	-19.9	1	12	1	4	3
		2		0	5	4
		3		1	6	4
		4		1	4	3
CAAUUUUAUAUGU	-20.5	1	13	1	6	3
		2		1	8	2
		3		1	6	1
		4		1	7	3
UGUUUGUAGU	-25.6	1	10	2	2	0
		2		1	3	2
		3		2	4	0
		4		2	3	1
UUGUCC	-24.0	1	6	3	1	1
		2		1	0	1
		3		0	0	0
		4		3	3	0
UAAAGUCUUCUA	-24.3	1	12	1	6	0
		2		0	5	0
		3		2	7	1
		4		1	5	1
UCAAUUGCAGU	-36.1	1	11	3	6	2
		2		3	6	2
		3		3	5	2
		4		3	5	2

[a] The binding site that is present in four copies in the RNA used for binding assays (and inserted into a reporter transgene for in vivo assays).

[b] Predicted ΔG for folding of entire RNA.

[c] Copy number refers to position of individual binding sites within the RNA.

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Chapter 3: BREs mediate both repression and activation of *oskar* mRNA translation and act in trans

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ABSTRACT

Asymmetric positioning of proteins within cells is crucial for cell polarization and function. Deployment of Oskar protein at the posterior pole of the Drosophila oocyte relies on localization of the *oskar* mRNA, repression of its translation prior to localization, and finally activation of translation. Translational repression is mediated by BREs, regulatory elements positioned in two clusters near both ends of the *oskar* mRNA 3' UTR. Here we show some BREs are bifunctional: both clusters of BREs contribute to translational repression, and the 3' cluster has an additional role in release from BRE-dependent repression. Remarkably, both BRE functions can be provided in trans by an *oskar* mRNA with wild type BREs but itself unable to encode Oskar protein. Regulation in trans is likely enabled by assembly of *oskar* transcripts in cytoplasmic RNPs. Concentration of transcripts in such RNPs is common, and trans regulation of mRNAs may therefore be widespread.

INTRODUCTION

Formation of the body plan of the Drosophila embryo relies on the action of several localized determinants [reviewed in (Lipshitz and Smibert, 2000; Palacios and St. Johnston, 2001)]. One of these, the Oskar (Osk) protein, is localized to the posterior pole of the oocyte and initiates formation of the pole plasm, which is responsible for posterior body patterning and germ cell formation. In the absence of Osk, both processes fail (Lehmann and Nüsslein-Volhard, 1986). Conversely, overexpression of Osk posteriorizes the embryo: a low level causes anterior patterning defects, while higher levels lead to replacement of all head and thoracic segments with a mirror image duplication of

posterior abdominal segments, the bicaudal phenotype (Smith et al., 1992). Similarly, specific misexpression of Osk at the anterior efficiently produces bicaudal embryos (Ephrussi and Lehmann, 1992). Thus, proper deployment of Osk is a critical step in embryonic pattern formation.

Several mechanisms are used to ensure that Osk appears at the appropriate level and only at the correct position at the posterior pole of the oocyte. Two such mechanisms act on *osk* mRNA. The first is mRNA localization (Kim-Ha et al., 1991; Ephrussi et al., 1991). At the early stages of oogenesis *osk* mRNA is efficiently transported from the nurse cells to the oocyte. During stage 9 *osk* mRNA is localized to the posterior pole. The second mechanism is translational control. Translation of *osk* mRNA is repressed prior to its localization, and then activated when posterior localization is achieved (Kim-Ha et al., 1995; Rongo et al., 1995; Markussen et al., 1995).

Translational repression of *osk* mRNA is mediated by sequences in the *osk* mRNA 3' UTR called BREs (Bruno Response Elements), which are bound by the Bruno (Bru) protein. The BREs are clustered in two regions, called AB and C. The AB region is near the *osk* open reading frame, while the C region is close to the polyadenylation site. Mutation of the BREs greatly reduces Bru binding in vitro and leads to precocious Osk protein expression in vivo, implicating Bru as a translational repressor (Kim-Ha et al., 1995; Webster et al., 1997). Repression must be released upon localization of *osk* mRNA. How this occurs is unknown, although two activating elements have been identified. One lies within the coding region near the 5' end of the *osk* mRNA (Gunkel et al., 1998). The second element is the IBE (Dimp Binding Element), a short sequence present in multiple copies throughout the *osk* mRNA 3' UTR (Munro et al., 2006).

Here we show that BREs have two roles, not one. Either of the two clusters of BREs mediates translational repression of unlocalized mRNA. The C region cluster of

BREs has an additional role in translational activation. Remarkably, both repressive and activating BRE functions can be provided in trans by *osk* transcripts with wild type BREs. We propose that *osk* RNP particles enable cross regulation between transcripts.

RESULTS

BREs have both positive and negative roles in control of Osk activity

Analysis of BREs provided evidence of translational regulation of *osk* mRNA. Mutation of BREs in both AB and C regions of an *osk* transgene (*osk ABC*$^{-}$) resulted in precocious expression of Osk protein and disruption of embryonic body patterning (Kim-Ha et al., 1995). We expected that the two clusters of BREs make additive or redundant contributions to repression. This is indeed the case, but selective mutation of subsets of the BREs reveals a novel activity. Transgenes were constructed in which only AB or C region BREs were mutated. All transgenes, including the original *osk ABC*$^{-}$, were introduced into the *osk*^{A87}*/Df(3R)osk* genetic background, in which endogenous *osk* mRNA is absent (Jenny et al., 2006), and tested for their ability to support embryonic body patterning (Fig. 3.1A).

The wild type *osk* transgene (*osk*$^{+}$) provides full Osk activity, and only wild type embryos are found (Fig. 3.1A). Mutation of just the AB region BREs (*osk AB*$^{-}$) does not substantially alter the patterning activity of the *osk* transgene, as almost all of the embryos have wild type segmentation (Fig. 3.1A). However, a very small fraction of embryos have anterior patterning defects, indicating of a low level of excess Osk and an extremely mild disruption of translational repression.

In contrast, mutation of just the C region BREs (*osk C*$^{-}$) dramatically reduces patterning activity of the transgene (Fig. 3.1A), with no corresponding reduction in

mRNA level (Fig. 3.1B). Thus, mutation of the C region BREs reveals a previously undetected positive role for BREs in control of Osk activity, which we show below to be translational activation.

We also tested the transgene in which BREs from both AB and C regions were mutated. This transgene produces excess Osk activity, consistent with the established role for the BREs in translational repression (Fig. 3.1A). This phenotype is not due to elevated mRNA levels (Fig. 3.1B). When taken together with the properties of the *osk AB*$^{-}$ and *osk C*$^{-}$ mutants, the phenotype of the *osk ABC*$^{-}$ mutant has two implications. First, the AB and C region BREs make redundant contributions to repression. Second, the requirement for the C region BREs in positive control of *osk* expression is reduced or eliminated when repression of *osk* is defective.

Based on the embryonic patterning phenotypes of the mutant *osk* transgenes we hypothesize (i) that Bru/BRE-dependent translational repression of unlocalized *osk* mRNA relies on contributions from both AB and C region BREs, and (ii) that the positive role of C region BREs is in activation of *osk* mRNA translation. This activation is required when all or a subset of BREs are intact and conferring repression.

Both AB and C region BREs contribute to translational repression

To test the prediction that the two sets of BREs act redundantly in translational repression, Osk protein accumulation was monitored in stage 8 oocytes (all assays were performed in the RNA null *osk*^{A87}*/Df(3R)osk* background). At this and earlier stages of oogenesis *osk* mRNA is highly concentrated in the oocyte. However, translation is repressed in wild type oocytes (Fig. 3.2A). Each of the transgenes with at least a subset of the BREs intact (*osk*$^{+}$, *osk AB*$^{-}$, and *osk C*$^{-}$) also fails to direct accumulation of any

detectable Osk protein in stage 8 oocytes (Fig. 3.2B and data not shown). By contrast, the *osk ABC*$^{-}$ transgene does produce readily detectable Osk (Fig. 3.2C). The results from this direct test of Osk protein accumulation are fully consistent with the assays of Osk body patterning activity: only the *osk ABC*$^{-}$ transgene produces high levels of ectopic Osk activity, while the other transgenes produce little or none (Fig. 3.1A). Therefore, BREs in either the AB or C region are sufficient to confer translational repression.

A *GFP* reporter mRNA was used to confirm that both AB and C regions mediate translational repression. The *UAS-GFP* reporter transgene includes UAS transcriptional control elements, the *GFP* coding region, and the polyadenylation signal and flanking sequences from the *fs(1)K10* 3' UTR (but not the portion of the 3' UTR that directs transport to the oocyte). In combination with the *maternal alpha tubulin GAL4* driver (*matGAL4*), the *GFP* mRNA is expressed in the nurse cells of the ovary and produces a high level of GFP (Fig. 3.3A). The reporter was modified by addition of the *osk* AB or C regions. The AB region confers very strong repression of translation: GFP fluorescence in the ovary is dramatically reduced (Fig. 3.3B) despite somewhat higher mRNA levels than for the control reporter (Fig. 3.3D). The C region also confers repression (Fig. 3.3C), although it is less effective than the AB region. Thus both AB and C regions mediate repression.

C region BREs mediate activation of translation

The extremely low level of Osk protein patterning activity from the *osk* transcripts with mutated C region BREs suggests that the normal activation of Osk protein expression at the posterior pole of the oocyte does not occur. In wild type ovaries Osk protein initially appears at the posterior of the oocyte beginning at stage 9, with

increasing levels by stage 10. This time course of Osk appearance is reproduced by the wild type *osk* transgene (Fig. 3.4A and data not shown). Similarly, the *osk AB*$^{-}$ transcripts direct localization-dependent expression of Osk at the posterior pole of the oocyte (Fig. 3.4A). However, the *osk C*$^{-}$ transcripts are impaired in Osk synthesis (Fig. 3.4A). Only a tiny fraction of oocytes display the strong posterior Osk accumulation of wild type, while the vast majority have low or undetectable levels of Osk. Thus, the C region BREs are required for normal Osk expression at the posterior pole of the oocyte.

The reduction or absence of posterior Osk protein from mutated C region BREs is not due to a reduction in mRNA level (Fig. 3.1B) and must arise from either of two defects: a failure of posterior *osk* mRNA localization, or a failure of translational activation. To distinguish between these options, the distribution of transgene mRNAs was monitored by in situ hybridization. Localization of *osk C*$^{-}$ transcripts was, as for wild type, robust (Fig. 3.4B). Thus, the very substantial defects in posterior Osk expression are not due to mRNA localization defects; mutation of the C region BREs interferes with activation of *osk* mRNA translation.

Disruption of translational activation by mutation of the BREs has been interpreted to be due to loss of the BRE binding sites. However, the mutations could have fortuitously created a novel binding site, to which an unknown ovarian factor binds and inhibits translation. A very strong argument against the latter interpretation comes from functional analysis of additional Bru binding sites recently identified by in vitro selection experiments (B. Reveal et al., unpublished). Several of the new sites are found in the *osk* mRNA, and all are clustered with the BREs in the AB and C regions (Fig. 3.8). Mutation of the type II Bru binding sites in the C region interferes with activation of translation, just as for mutation of the C region BREs although to a somewhat lesser extent (Figs. 3.1A and 3.4A). Because the mutated BREs and type II sites are highly dissimilar, the

possibility that both types of mutations fortuitously caused the same type of artifactual translation defect seems implausible.

Translational activation by C region BREs is not required when repression is defective

Mutation of the C BREs alone leaves repression of the unlocalized mRNA intact (Fig. 3.2) but significantly disrupts activation with greatly reduced posterior Osk (Fig. 3.4A). When both AB and C BREs are mutated and repression is disrupted, then posterior accumulation of Osk protein is restored (Fig. 3.4A). These experiments illustrate why the activating role of the C region BREs was not detected in the initial analysis of BRE function, which relied on the *osk ABC*$^{-}$ transgene with both AB and C region BREs mutated (Kim-Ha et al., 1995).

Translational activation by the C region BREs does not affect poly(A) tail length

Cytoplasmic polyadenylation serves as one form of translational activation of *osk* mRNA (Chang et al., 1999; Castagnetti and Ephrussi, 2003). Repression by BREs does not involve alteration of the poly(A) tail (Lie and Macdonald, 1999; Castagnetti and Ephrussi, 2003), making it highly unlikely that activation by the BREs could involve cytoplasmic polyadenylation. However, the mechanism of activation by the IBEs is unknown. We evaluated relative poly(A) tail lengths of transgene mRNAs using a thermal elution assay (Simon et al., 1996) in which mRNAs bound via their poly(A) tails to poly(U) agarose are eluted stepwise at increasing temperatures. Transcripts with short tails elute at lower temperatures than those with longer tails. Notably, the elution profiles were indistinguishable for wild type *osk* transcripts and *osk* transcripts with translational

activation defects due to mutation of BREs (*osk C*$^{-}$) or IBEs (*osk IBE*$^{-}$) (Fig. 3.5A). In contrast, the elution profiles for wild type *osk* mRNA from *orb*$^{+}$ or *orb*$^{-}$ mutant ovaries were dramatically different (Fig. 3.5A), consistent with previous work (Chang et al., 1999; Castagnetti and Ephrussi, 2003). A PCR-based assay of poly(A) tail length also showed no substantial differences in the poly(A) tails of the *osk*$^{+}$, *osk C*$^{-}$ or *osk IBE*$^{-}$ transcripts (Fig. 3.5B).

Trans-regulation of *osk* mRNA translation

The above experiments with *osk* transgenes were all performed in the *osk* RNA null background, where the only *osk* mRNA present is that from the transgene. To ask if the misregulation of *osk* mRNA with mutant BREs can be influenced by *osk* mRNA with wild type BREs, these transgenes were also tested in the presence of the *osk*^{54} mRNA (the *osk*^{54} allele is protein null and provides no Osk protein activity, but retains the BREs and has wild type levels of the mutant RNA; some of these experiments were repeated with *osk*^{84}, which also retains the BREs and lacks both Osk protein activity and detectable Osk, with essentially identical results). Remarkably, the patterning defects caused by disruption of BRE-dependent repression or activation of *osk* mRNA translation are dramatically suppressed when *osk*^{54} mRNA is present.

The *osk ABC*$^{-}$ transgene is defective in translational repression, and produces substantial ectopic Osk protein activity in the *osk* RNA null background. When *osk*^{54} mRNA is present, the ectopic Osk is largely eliminated (Fig. 3.2D, Fig. 3.6). We also tested a transgenic line with higher levels of the *osk ABC*$^{-}$ mRNA (2x *ABC*$^{-}$)(Fig. 3.1B) and more severe patterning defects (Fig. 3.6 and legend). The extreme patterning

phenotype of this transgenic line is also suppressed when the *osk*^{54} mRNA is present, although suppression is incomplete (Fig. 3.6).

We next asked if the defect in translational activation of the *osk C*$^{-}$ transgene could also be suppressed by the presence of *osk* mRNA with wild type BREs. Strikingly, this transgene produces little or no Osk patterning activity in the absence of endogenous *osk* mRNA, yet it supports wild type body patterning in combination with the *osk*^{54} mRNA (Fig. 3.6). Although rescue of body patterning is dramatic, restoration of Osk protein expression in stage 10 oocytes is comparatively modest (Fig. 3.4A). A likely explanation comes from a consideration of the time course of Osk expression. Osk is first detected at the oocyte posterior pole at stage 9, with more present at stage 10. However, the bulk of Osk accumulation occurs later in oogenesis (Snee et al., 2007). Therefore, the contribution of the C region BREs to activation of *osk* mRNA translation, while detectable at stage 10, may be more significant later. If so, then it may be this later phase in Osk expression that is most effectively restored by coexpression of the *osk C*$^{-}$ transgene with the *osk*^{54} mRNA. Notably, embryos from mothers expressing only the *osk C*$^{-}$ transcripts have no detectable Osk, while coexpression with the *osk*^{54} mRNA partially restores embryonic Osk (Fig. 3.7). Thus, the later phase of Osk expression is most severely affected by disruption of BRE-dependent translational activation, and is most substantially restored by coexpression with an *osk* mRNA with wild type BREs.

As a separate assay for rescue of posterior Osk accumulation, we also determined the number of pole cells formed in embryos from mothers expressing the *osk C*$^{-}$ transgene, with or without the presence of the *osk*^{54} mRNA. Embryos from mothers expressing only the *osk C*$^{-}$ transgene had no pole cells, and coexpression with the *osk*^{54} mRNA partially restored pole cell formation (Fig. 3.7B).

Rescue of *osk* mRNA regulatory defects in trans is selective. There is no rescue at all for the *osk IBE*$^{-}$ transgene: Osk protein accumulation (Fig. 3.4A), Osk patterning activity (Fig. 3.6), and pole cell formation (Fig. 3.7) all remain defective when the *osk*^{54} mRNA is present.

DISCUSSION

Three types of regulatory elements have been implicated in activation of *osk* mRNA translation: a 5' activating element, the IBEs and now the subset of BREs in the *osk* 3' UTR C region. The BREs present an unusual case, being involved in both repression and activation. In principle, a repressive element could be thought to play a passive role in the activation that relieves repression: the element would need to be unoccupied or unproductively bound for activation to occur. For the BREs the role is active, not passive. In the context of the *osk C*$^{-}$ transgene, repression occurs because the AB region BREs are intact. However, despite proper localization of the *osk C*$^{-}$ mRNA to the posterior pole of the oocyte, the normal activation of translation to allow Osk protein expression at that site is defective and Osk protein levels are reduced. Thus, the C region BREs are required to release the mRNA from repression conferred by the AB region BREs. In the context of wild type *osk* mRNA, both AB and C region BREs contribute to repression, and so the C region BREs must switch roles, first repressing and later activating. The activating function of C region BREs could be due to position in the mRNA. For example, activation might only occur when BREs are close to the poly(A) tail. Given the absence of a change in poly(A) tail length when activation is defective, any effect on the poly(A) tail itself would have to be more nuanced under this scenario. Activation could involve cooperation between the BRE-binding factor and another

activating factor that binds only in the C region. At present, no protein is known to have that property.

The best candidate for the factor that binds to the BREs to mediate activation is Bru, since the BREs were identified and defined by their ability to bind Bru. Moreover, mutation of the type II Bru binding sites in the C region also disrupts activation. Therefore, if the activator is not Bru, it must be a protein or proteins with the ability to bind to the two different types of sites. Mutants lacking Bru function arrest oogenesis at a very early stage (Schupbach and Wieschaus, 1991; Webster et al., 1997), obscuring any potential role in activation of *osk* mRNA translation.

Certain defects in translational regulation of *osk* mRNA can, remarkably, be suppressed by the presence of an *osk* mRNA with wild type regulatory elements. This novel phenomenon is reminiscent of transvection, in which regulatory elements controlling transcription of one allele of a gene can influence transcription of the second allele on the homologous chromosome (Lewis, 1954; Duncan, 2002). We suggest a similar model for translational regulation in trans, in which regulation imposed on one molecule via direct binding of regulatory factors is then conferred on another molecule via association of the mRNAs. Evidence for a physical association between *osk* transcripts has come from the demonstration that reporter mRNAs containing the *osk* 3' UTR (which is necessary but not sufficient for localization) localize to the posterior pole of the oocyte only if endogenous *osk* mRNA is also present (Hachet and Ephrussi, 2004). This 'piggybacking' of the reporter mRNA relies on the PTB protein. PTB binds to multiple sites in the *osk* mRNA, forming a large aggregate in vitro. Thus, it appears that PTB links multiple *osk* transcripts to form large RNP particles in vivo (Besse et al., 2009). Piggybacking for mRNA localization provides an example of a trans effect in post-transcriptional regulation. For piggybacking all that is necessary is the physical

linkage: directed movement of one *osk* mRNA molecule to its destination at the posterior pole of the oocyte would confer the same movement on any other molecule in the same RNP particle.

Would physical linkage alone be sufficient to confer all of the different forms of translational regulation on all *osk* mRNAs in the same RNP particle? At least one type of regulation – activation by the IBEs – is not conferred in trans, providing an example where physical linkage is not sufficient. However, under the current models for Bru/BRE-dependent repression, physical linkage could be sufficient for trans regulation by BREs. One model for repression involves the formation of silencing particles which in some manner limit accessibility to ribosomes (Chekulaeva et al., 2006). Presumably, any mRNA recruited to the particles would also be protected from ribosomes. A second model for repression involves recruitment of Cup to the *osk* mRNA by Bru. Cup binds to, and inactivates, eIF4E, thus interfering with initiation of translation. If the inactivated molecule of eIF4E is bound to the *osk* mRNA cap, then translation initiation is blocked (Nakamura et al., 2004). A weak point of this model has been the necessity that, for repression to be specific, both RNA contacts of the Bru/Cup/eIF4E complex would have to be with the same mRNA molecule: eIF4E would have to bind the cap of a particular *osk* transcript, and Bru would have to bind the BREs of the same transcript. What would prevent the Cup newly recruited by Bru to *osk* mRNA from inactivating the eIF4E bound to the cap of a different mRNA? In the context of an RNP containing predominantly *osk* mRNAs, inactivation of eIF4E by Cup would interfere with translation of any member of the local population of transcripts, even if the Bru/Cup/eIF4E ternary complex bridges two mRNAs. By this scenario, trans regulation would be an inherent feature of the mechanism. The specificity of such trans regulation would be limited by the degree to which the local population of transcripts is homogeneous, and cross regulation between

different species of mRNAs would be possible. Recent characterization of sponge bodies has shown that *osk* mRNA is compartmentalized in the oocyte, with large reticulated sponge bodies having *osk* distributed in discrete domains (Snee and Macdonald, 2009). Compartmentalization of *osk* mRNA could impose selectivity on trans regulation, preventing features of *osk* regulation from being conferred promiscuously on other mRNAs. Assembly of mRNAs in large RNP particles is common, and elucidation of the rules dictating which types of translational regulation can and cannot be exerted in trans should have broad relevance.

MATERIALS AND METHODS

Flies and transgenes

w1118 flies were used as the wild type. Mutant flies, including osk^{54}, osk^{84}, osk^{A87}, orb^{MEL}, and orb^{DEC}, are described at FlyBase (http://flybase.bio.indiana.edu/). *Df(3R)osk* was constructed by FRT-mediated recombination (Parks et al., 2004) using Exelixis transposon insertions P(XP)d09940 and PBac(WH)f02664 (Thibault et al., 2004). The resulting deletion of 23,158 nt removes four genes: *osk*, CG11963, CG11964 and CG11966. Flies bearing *osk* transgenes with mutation of IBEs, either all 13 IBEs or just the 5' subset of 3 IBEs (Munro et al., 2006), were from Bruce Schnapp. The *osk*+ and *osk ABC*- transgenes were described previously (Kim-Ha et al., 1995), and carry the mutations described there (and also below). Novel *osk* transgenes described here include the following mutations (underlined in the sequences below). For mutations in the AB and C regions the portions of the relevant *osk* genomic sequence (GenBank Accession M63492) are provided: AB, nt 2669-2795; C, nt 3397-3555.

AB-:

GAATTCGCTTAGTTTTAATTAGTTTTTAATTGAGATTGTTCTCTGTCTTTGTTA
TTTTAGATTTTCGTGCACTTGTCCTAGTCCATTATTTTAGATTATTTTGGGTTT
TGGTTTCTTAGTTAGATTTAAA

C-:

TTCTGGCGTAATTTACAGCTCTACTTTAAAGTCTTCTAGATAGCTATCTACTAT
TTATAAACTTATTTATTGTCTTGAATTTGAGTTAACTTGAGTTATTGATGGTGA
TCACGTTTTTTTTGTCCTATAACAAGCTGCAATGTAAAATCCAAAAAA

AB II-:

GAATTCGCTTAGTTTTAATATGTTTTATATGTAGTATGTTCTCTGTCTTTGTTT

ATTTATATGTTCGTGCACTCGCTTTAGTCCATTATTGTATATTATTGTGTGTTT

TGTGTTCTATGTTAGATTTAAA

C II-:

TTCTGGCGTAATTTACAGCTCTACTTTAAAGTCTTCTAGATAGCTATCTACTAT

TTATAAACTTATTTATCGCTTTGAATGTATGTTAATTGTATGTATTGATGGTGA

TCACGTTTTTTTCGCTTTATAACAAGCTGCAATGTAAAATCCAAAAAA

C III-:

TTCTGGCGTAATTTACAGCTCTACTTATACTATCGTATGATAGCTATCTACTAT

TTATAAACTTATTTATTGTCTTGAATGTATGTTAATTGTATGTATTGATGGTGA

TCACGTTTTTTTTGTCCTATAACAAGCTGCAATGTAAAATCCAAAAAA

UAS-GFP has mGFP6 (Haseloff, 1999) inserted into the Asp718 site of pUASp (Rorth, 1998). The AB and C regions of the *osk* 3' UTR [nt 2669-2795 and 3397-3555, respectively, of the *osk* genomic sequence (GenBank Accession M63492)] were inserted as BamHI-BglII fragments into the BamHI site from the pUASp vector.

Analysis of proteins and mRNAs

Immunostaining of ovaries and embryos was as described previously (Kim-Ha et al., 1995), except that secondary antibodies were labeled with Alexafluor 488 (Invitrogen). Osk was detected with rabbit anti-Osk (diluted 1:2,000) and Vas was detected with rat anti-Vas (1:500). Fluorescent in situ hybridization was as described (Snee and Macdonald, 2009). In all cases *osk*+ controls were fixed, stained and imaged in

parallel, to confirm that the staining worked properly and to provide a reference for determination of signal levels. Microscopy of all samples made use of a Leica TCS-SP laser scanning confocal microscope or a Nikon epifluorescence microscope. For RNase protection assays ovaries were dissected from females maintained with males on well yeasted vials for 3-4 days after eclosion, and RNA prepared using Tri Reagent-LS (Molecular Research Center, Inc) following the protocol provided by the vendor. RNase protection assays were performed with the RPA III kit (Ambion, Inc) and the results quantitated using phosphorimaging with a Typhoon Trio Imager (Amersham). Probes for RNase protection were transcribed in vitro using the Maxiscript kit (Ambion).

The thermal elution assay was used largely as described (Simon et al., 1996), with some modifications. Poly (U)-Sepharose (Sigma) was swollen in 2 M NaCl and 5 mM Tris-HCL, pH 7.5, washed extensively in EB buffer (90% formamide, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.2% SDS), and equilibrated in CSB15 buffer (15% formamide, 700 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA). RNA samples (100 ug of ovarian RNA) were suspended in 50 μl of 1% SDS, 30 mM EDTA, heated at 70°C for 5 min, and then diluted 5 fold in CSB15 buffer. The RNA was mixed with 50 μl of gravity packed beads in a 500 μl microfuge tube, which was rotated at room temperature for 30 min. After a short, low speed centrifugation, the supernatent was recovered (this was the 25C° sample) and the beads were washed three times with LSB15 buffer (15% formamide, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA). 200 μl of LSB15 buffer was added and the beads were incubated at 30°C for 4 min in the heating block of a PCR machine. The beads were again pelleted by a short centrifugation, and the supernatent recovered (the 30°C sample). Washing and thermal elution steps were repeated at temperatures up to 65°C. RNA samples were diluted with 0.1% SDS, extracted with phenol/chloroform and ethanol precipitated.

The LM-PAT assay was performed essentially as described (Salles et al., 1994) using ovarian RNA prepared as described above. The oligo dT anchor primer was GAGCTCATTTGCGGCCGCTTTTTTTTTTTT and the *osk* specific primer was GTCTTCTAGATAGCTATCTAC.

UV crosslinking

UV crosslinking with ovarian extracts was performed as described previously (Kim-Ha et al., 1995), except that phosphorimaging was used for signal detection. The AB and C RNA probes were either wild type or had the mutations indicated in Fig. 3.8 and correspond to nt 2669-2795 (AB) or 3397-3602 (C) of the *osk* genomic sequence (GenBank Accession M63492).

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FIGURES

Figure 3.1. Body patterning activity of *osk* transgenes.

A. Transgenes were tested in the osk^{A87}/*Df(3R)osk* background, and progeny embryos examined for cuticular phenotypes. Categories of phenotypes are arranged with the lowest levels of Osk patterning activity at left and the highest levels at right.

B. Transcripts levels for *osk* trangenes. All transgenes were tested in the osk^{A87}/*Df(3R)osk* background, such that the only *osk* mRNA present is from the transgene. Top: RNase protection assays of *osk* and *rp49* mRNAs from ovaries of the indicated genotypes. Bottom: Levels of transgene mRNAs relative to the level of endogenous *osk* mRNA in wild type (*w1118*) flies. RNA levels were quantified by phosphorimaging and *osk* levels normalized using the *rp49* signal. Three or more assays were used to generate the average levels (and standard deviations). Flies had one copy of the transgene-bearing chromosome. The translational activation defects of the *osk* C II- and osk C III- transgenes (panel A, see also Fig. 3.4) are not due to low mRNA levels. In addition, the severity of the activation defects may be underrepresented relative to the *osk* C- transgene (Fig. 3.1), as the levels of the *osk* C II- and *osk* C III- mRNAs are higher than for *osk* C-.

A

transgene	Cuticular phenotype (%) 0-1 abd. segments	2-7 abd. segments	wt	head defects	loss of head and thoracic segments	bicaudal	n
osk^{+}	-	-	100	-	-	-	344
osk AB^{-}	-	-	96	4	-	-	236
osk AB II^{-}	-	-	100	-	-	-	354
osk C^{-}	83	14	3	-	-	-	444
osk C II^{-}	100	-	-	-	-	-	132
osk C III^{-}	<1	<1	99	-	-	-	412
osk ABC^{-}	-	1	4	24	49	21	340
osk IBE^{-}	100	-	-	-	-	-	643

B

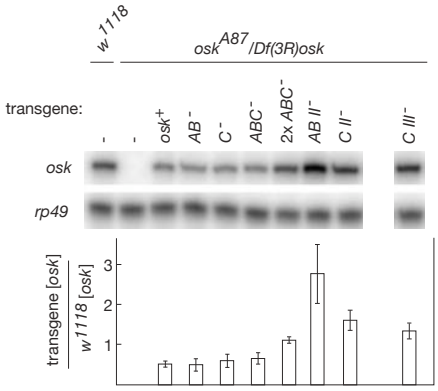


Figure 3.2. Additive contributions of BREs to translational repression.

Panels A-D show stage 8 egg chambers with Osk (green) detected by immunofluorescence and nuclei (red) stained with ToPro. A is wild type and B and C are *osk*^{A87}/*Df(3R)osk* with the *osk* AB- (B) or *osk* ABC- (C) transgenes. Results similar to that shown in B were obtained for the wild type and *osk* C- transgenes. D is *osk*^{54}/*Df(3R)osk* with transgene *osk* ABC-. All egg chambers show a low level of green signal in both germline and somatic cells, which is due to background staining by the antibody. The only consistent difference among the different genotypes was the higher level of Osk in the oocytes of *osk*^{A87}/*Df(3R)osk* ovaries with the *osk ABC-* transgene present. This ectopic Osk was always present uniformly throughout the oocyte cytoplasm, and was detected at roughly the level shown in 69% (n=18) of the egg chambers expressing *osk ABC-*, with many of the remaining egg chambers showing a lower level but still above background. In contrast, none of the other genotypes ever showed Osk above background.

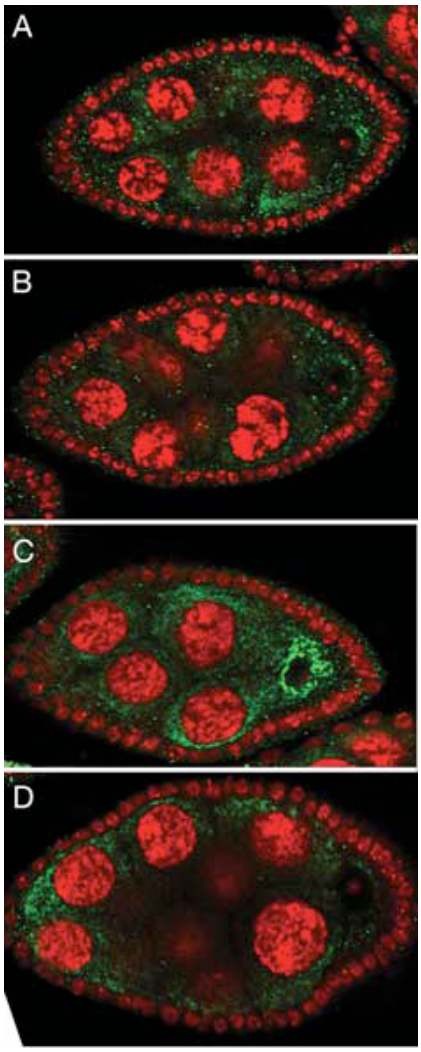


Figure 3.3. Both AB and C regions of the *osk* mRNA 3' UTR confer translational repression on reporter mRNAs.

Panels A-C show stage 10A egg chambers expressing a GFP transgene with GFP in green and nuclei stained with ToPro in red. All samples were fixed in parallel and imaged together with the same laser power and confocal settings. A is UAS-GFP, B is UAS-GFP- AB, and C is UAS-GFP-C, where AB and C are the eponymous regions of the *osk* 3' UTR. Panel D shows RNase protection assays of mRNA levels of the transgenes. *rp49* is used as a loading control.

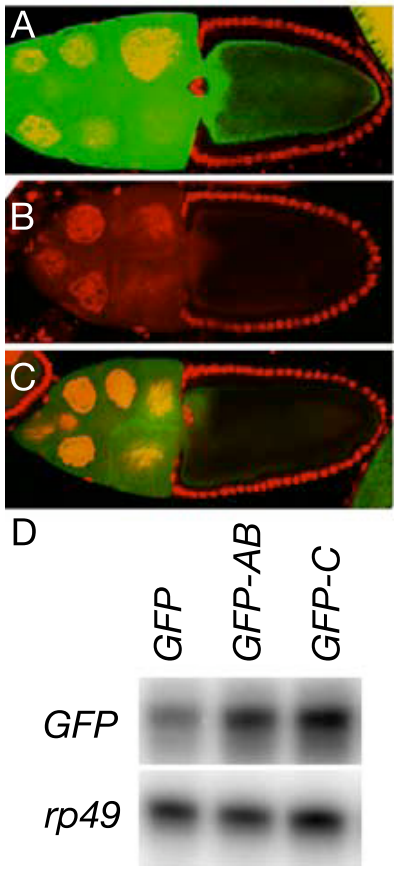
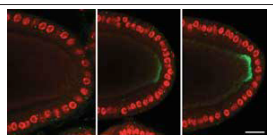


Figure 3.4. Mutation of C region BREs inhibits accumulation of Osk protein at the posterior pole of the oocyte, but not *osk* mRNA localization.

A. Osk protein in oocytes. Shown at top are posterior portions of stage 10A egg chambers, with examples of the different levels of Osk detected by immunofluorescence. Osk in the oocyte is green, and the somatic follicle cell nuclei are red (ToPro staining). The fraction of stage 10A egg chambers showing each of the different levels of Osk is indicated below for each *osk* transgene. Transgenes were in the osk^{A87}/*Df(3R)osk* background, except for those at the bottom in which osk^{54} was present (as indicated). The *osk IBE-* transgenes have mutations of the first three IBEs (Munro et al., 2006), and the results with the osk IBE- transgene reproduce the results of (Munro et al., 2006). The *osk ABC-* transgene only poorly rescues the oogenesis progression defects of the osk^{A87}/*Df(3R)osk* mutant, and even those egg chambers that develop to later stages often display morphological abnormalities (data not shown). When an additional copy of this transgene is provided, rescue of the oogenesis defects and morphological abnormalities is more complete and all stage 10A egg chambers have strong posterior Osk. Thus, it is likely that the absence of posterior Osk in a small fraction of egg chambers with only a single copy of the *osk ABC-* transgene is a secondary consequence of the poor rescue of progression through oogenesis (the *osk* RNA null phenotype).

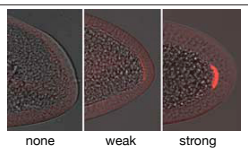
B. *osk* mRNA in oocytes. Shown at top are posterior portions of stage 10A egg chambers, with examples of the different degrees of posterior *osk* mRNA localization detected by fluorescent in situ hybridization. The *osk* mRNA signal is red. The fraction of stage 10A egg chambers showing each of the different levels of posterior *osk* localization is indicated below for each *osk* transgene. All transgenes were in the osk^{A87}/*Df(3R)osk* background. The absence of localization for a minor fraction of the *osk ABC-* egg chambers is likely due to the incomplete rescue of the oogenesis progression defects by this transgene, as explained above.

A
Osk protein



	none	weak	strong	n
osk^{+}	-	2%	98%	48
osk AB^{-}	-	-	100%	36
osk AB II^{-}	-	-	100%	121
osk C^{-}	10%	88%	2%	52
osk C II^{-}	5%	56%	38%	97
osk C III^{-}	-	19%	81%	16
osk ABC^{-}	15%	-	85%	41
osk IBE^{-}	100%	-	-	33
osk C^{-} + osk^{54}	2%	70%	28%	51
osk IBE^{-} + osk^{54}	100%	-	-	27

B
osk mRNA



	none	weak	strong	n
osk^{+}	-	4%	96%	24
osk C^{-}	-	-	100%	20
osk C II^{-}	-	-	100%	20
osk C III^{-}	-	-	100%	20
osk ABC^{-}	22%	-	78%	14

Figure 3.5. Translational activation defects and poly(A) tail length.

A. Ovarian RNAs were purified, bound to poly U agarose, and eluted at the temperatures indicated. Each fraction was tested by RNase protection assay for the RNA indicated. As indicated, the *osk* transgenes were tested in the osk^{A87}/*Df(3R)osk* background. The eluted fractions of RNA from *orb* mutant ovaries (orb^{MEL} / orb^{DEC}) were tested for both *osk* and *rp49* to confirm that polyadenylated mRNAs were indeed bound to the poly U agarose.

B. Ovarian RNAs from osk^{A87}/*Df(3R)osk* females expressing the indicated transgenes were subjected to the PAT assay (Salles et al., 1994). The distribution of the signal in each lane reflects the range of poly(A) tail length.

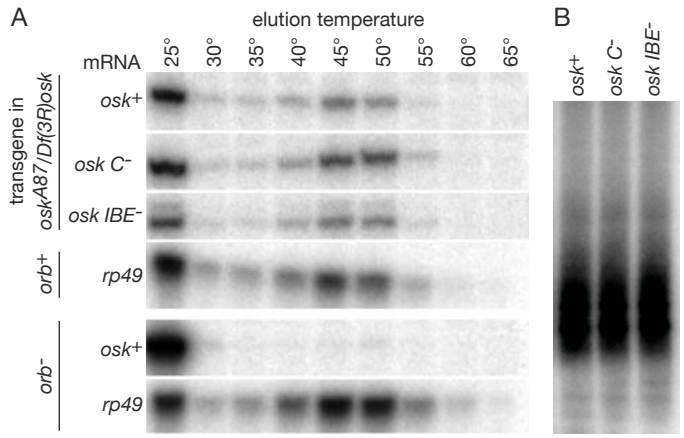


Figure 3.6. Suppression of regulatory defects by BRE+ mRNA.

Body patterning activities of *osk* transgenes in the absence of endogenous *osk* mRNA (upper part) or in the presence of the BRE+ *osk*^{54} mRNA (lower part). The transgenes are indicated at top. For each transgene the percentage of progeny embryos with different levels of Osk activity is indicated in the graph below (shading key at bottom). Levels of Osk activity: low/none, missing or absent abdominal denticle belts; wild type, wild type cuticles; excess, loss of anterior structures or bicaudal phenotypes. 2x *osk ABC*- is a transgenic line that expresses twice the level of the line used in all other experiments and appears to have two insertions (data not shown). Embryos in the 'excess' category for the 2x line typically have more extreme phenotypes than for the 1x line (e.g. 56% bicaudal for 2x vs 13% bicaudal for 1x). The *osk IBE*- transgene has the 5' subset of the IBEs mutated [subset A in (Munro et al., 2006)]. A similar transgene with all IBEs mutated (Munro et al., 2006) yielded identical results (data not shown).

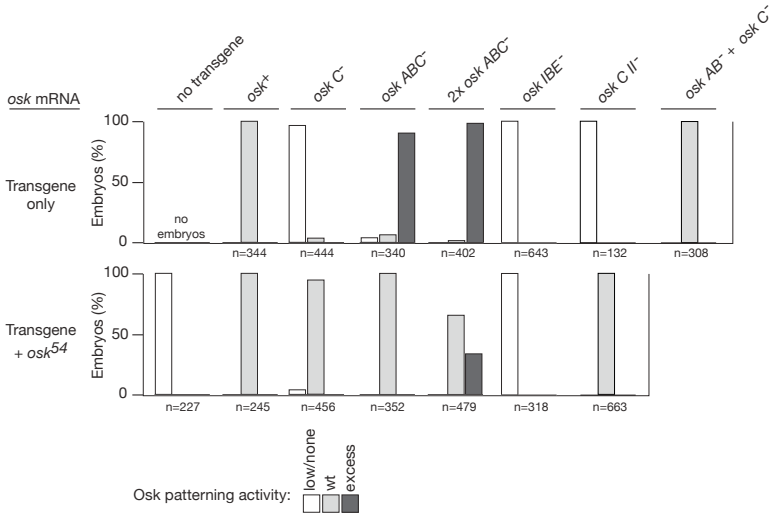
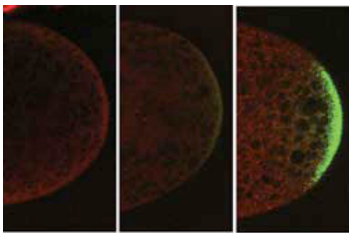


Figure 3.7. Rescue of translational activation by BRE+ mRNA.

A. Osk protein in embryos from mothers expressing *osk* transgenes, with or without the BRE+ *osk*^{54} mRNA. Shown at top are posterior portions of early stage embryos, with examples of the different levels of Osk (green) detected by immunofluorescence. For each transgene the percentage of progeny embryos with different levels of Osk protein is indicated below.

B. Pole cell numbers for embryos from mothers expressing the transgene indicated at top, with or without *osk*^{54} mRNA as indicated. The results in this Figure confirm that the translational activation defect of the *osk C* and *osk C II-* mRNA is largely suppressed by the *osk*^{54} mRNA, as revealed both by direct examination of Osk protein and the sensitive biological assay of pole cell formation. The *osk C III-* transgene displays a translational activation defect as measured both by Osk protein and pole cell formation. This defect is not readily apparent in the body patterning assay (Fig. 3.1A), for which a low level of Osk will suffice. Curiously, there is no substantial suppression of the *osk C III-* defects by *osk*^{54} mRNA; the *osk C III-* mutation behaves like the IBE mutations in this regard. The *osk C III-* site is positioned between closely spaced IBEs; perhaps this mutation affects IBE function through a change in local mRNA structure.

A Osk protein



Transgene only	none	weak	strong
osk^{+} (n=18)	-	6%	94%
osk C^{-} (n=14)	100%	-	-
osk C II^{-} (n=17)	100%	-	-
osk C III^{-} (n=19)	68%	32%	-
osk IBE^{-} (n=14)	100%	-	-
Transgene + *osk*^{54}			
osk^{+} (n=19)	-	11%	89%
osk C^{-} (n=19)	26%	63%	11%
osk C II^{-} (n=17)	50%	44%	6%
osk C III^{-} (n=19)	60%	20%	20%
osk IBE^{-} (n=16)	100%	-	-

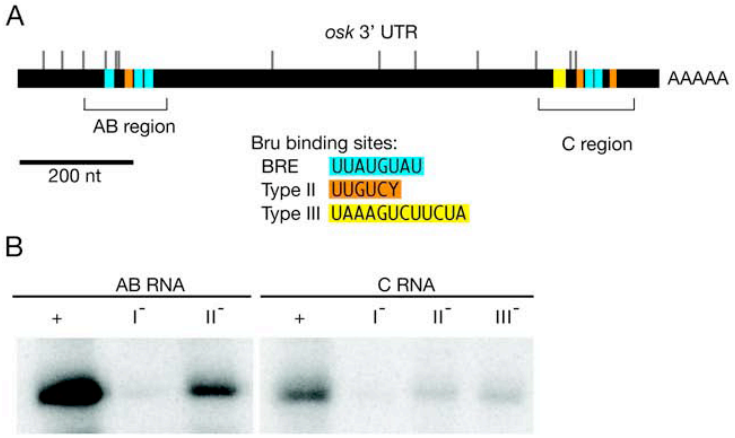
B Pole cell number

	Transgene only	Transgene + *osk*^{54}
osk^{+}	18.5 (n=26)	17.4 (n=22)
osk C^{-}	0 (n=15)	12.9 (n=23)
osk C II^{-}	0 (n=19)	8.3 (n=23)
osk C III^{-}	3.9 (n=22)	4.1 (n=18)
osk IBE^{-}	0 (n=12)	0 (n=14)

Figure 3.8. Novel Bru binding sites and their functional analysis.

A shows a diagram of the *osk* 3' UTR, with known regulatory and binding sites indicated. The IBEs are shown as vertical lines above the horizontal bar. The IBE and BRE sites are described in the text. The *osk IBE-* transgene has mutations of the first three IBEs. The type II and III Bru binding sites were identified by in vitro selection and their sequences are shown.

B. UV crosslinking assay of Bru binding to AB and C region RNAs bearing mutations in different classes of Bru binding sites. Mutation of the BREs has the strongest effect, mutation of the type II sites has a lesser effect, and the weakest (but still reproducible) effect comes from mutation of the type III site. Equal amounts of radiolabeled RNA were used for each assay.



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